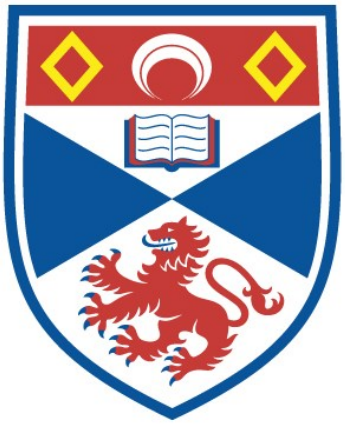
PERMEABILITY AND RESPIRATION EFFECTS OF THE THYROIDAL HORMONES IN BUFO BUFO (L)

Keith Green

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1964

PERMEABILITY AND RESPIRATION EFFECTS OF THE THYROIDAL HORMONES IN Bufo bufo (L).

by

Keith Green, B.Sc. (Leics.).

THESIS

presented for

the Degree of Doctor of Philosophy in the Faculty of Science of the University of St. Andrews.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

DECLARATION

I hereby declare that the work recorded in this Thesis has been carried out by myself, and that it is of my own composition. I further declare that it has not been submitted in any previous application for a Higher Degree.

RESEARCH CAREER

I graduated in Zoology from the University of Leicester obtaining a B.Sc. (Honours). The research work recorded in this Thesis has been carried out during the nine terms between August, 1961 and November, 1963, during which time I held a Nuffield Studentship in the University of St. Andrews.

SUPERVISORS CERTIFICATE

I certify that Keith Green has fulfilled the conditions laid down in the regulations for a Degree of Ph.D., under Ordinance No. 16 of the University Court of the University of St. Andrews and that he has accordingly qualified to submit this Thesis for the Degree of Doctor of Philosophy.

ACKNOWLEDGEMENTS

I wish to thank my supervisor Dr. A.J. Matty most sincerely for his unfailing encouragement and advice during the course of the work reported here. Also, I wish to thank Professor R.B. Hunter for the hospitality of his department, and all the members of the Gatty Marine Laboratory for the hospitality and kindness extended to me during the tenure of my Studentship.

CONTENTS

SECTION I

SECTION II

SECTION III

Page

Effect of thyroid hormones on the ionic permeability of the isolated toad bladder and skin.

SECTION IV

Effects of thyroidal hormones on the oxygen consumption of isolated toad tissues.

SECTION I

GENERAL INTRODUCTION

a. Concepts of hormone action.

Since the time that hormones were first identified, and later purified, many investigations have been performed in attempts to determine their mode of action in the living animal. Much of this work initially concentrated on effects within the whole animal. This was undertaken either by extirpation of the gland which secreted the hormone under investigation, and subsequent observation of effects caused by this procedure, or by injection of the purified hormone. With improvement of techniques and development of apparatus, work on isolated tissues and, further, on sub-cellular particles has been performed, and these technical advances have encouraged great strides in our understanding of hormone action.

Original concepts of hormone action have undergone much alteration and work during more recent years has shown that many hormones, differing in molecular size and shape as well as in chemical structure, may affect similar processes in cells. This wind of change was begun in the early 1940's by the work of C. L. Gemmill (1940, 1941) who showed that insulin increased muscle uptake of glucose. Immediately after this discovery, Green (1941) made the suggestion that hormones may work by influencing specific rate-limiting enzymes. This concept was in keeping with the general atmosphere prevailing at this time when metabolic systems were being described in detail. As soon as certain enzymes were

isolated, many investigators inevitably searched for effects of hormones on individual enzymes and enzyme systems. These theories, however, were inconclusive, for they left many well known observations unexplained. Shortly afterwards, it became evident that the only way to obtain meaningful observations of hormone effects was by using a completely organised cell, and not by utilising broken cell preparations (Levine and Goldstein, 1955). This view developed as the biochemists realized that the living cell had a meaningful architecture, and that spatial relationships within the cell played an important role in cellular functions.

Thus the concept was developed that cellular activity can be stimulated to a higher level by providing more substrate for the existing enzymes rather than by directly influencing any enzymes in the cell. Subsequent work along similar lines by Levine and his colleagues (1949, 1950), confirmed by Park, Johnsen, Wright and Bastel (1957), led to the view that insulin acts on the glucose transfer system which is located in the cell membrane. All the work leading to this concept has been reviewed by Levine (1957) where the action of insulin, in particular, is discussed with reference to its effect of increasing the intracellular concentration of sugars, an effect independent of any enzymic system. This concept of a hormone-membrane interaction has been expanded to apply to a number of hormones in the light of results obtained by a variety of techniques. A number of such results are outlined below, and illustrates the diversity of the hormones which affect permeability.

Vasopressin increases both water retention and water uptake through the skin of whole adult Amphibia (Heller, 1945) and, in addition, when the hormone is directly applied to the serosal surface of skin *in vitro*, it stimulates active sodium transport (Fuhrman and Ussing, 1951; Ussing and Zerahn, 1951). This hormone also accelerates water movement down an osmotic gradient, measured gravimetrically, across the isolated urinary bladder of the toad *Bufo marinus*, (Bentley, 1958; Leaf and Hays, 1961), in addition to stimulating active sodium transport under conditions of no osmotic gradient as measured both electrically (Bentley, 1960), and isotopically (Leaf, 1961). Aulsebrook (1961), by addition of a vasopressin preparation to the serosal surface of rat colon, obtained an increase in net sodium transfer to this surface of the membrane. Crabbé (1961 a,b) has shown that active sodium transport across the bladder of *Bufo marinus* is increased either by previous injection of aldosterone or direct application of the hormone to the membrane. By measuring the effects of cortisol and certain synthetic steroids on the permeability of the rabbit synovial membrane to dyestuffs Sharp (1963) found that all the compounds decreased the permeability to the same extent, and that they all acted within a short time after injection. It has been reported by Leaf (see Goodfriend and Kirkpatrick, 1963) that insulin causes a slight increase in sodium transport in the bladder membrane, and Herrera, Whittembury and Planchart (1963) have shown that a similar response to insulin is found in frog skin. Although Rasmussen, Schwartz, Young and Marc-Aurele (1963) have shown that, even at high concentrations of insulin, there is no effect on water flow across the isolated

bladder down an osmotic gradient. In addition, Beigelman and Hollander (1962) and Zierler (1956) have reported increases in resting potentials of rat epididymal adipose tissue and rat skeletal muscle respectively, after insulin treatment. The effects of insulin on the transfer of sugars across muscle cell membranes are well known from the work of Randle and Morgan (1962) and Krahl (1961). Barnett and Ball (1960) obtained direct evidence of an effect of insulin on membranes from their electron microscope studies of rat epidioymal tissue. Upon addition of insulin profound morphological changes occur, the plasma membranes of the cells become invaginated at many sites and it is apparent that pinocytosis is initiated.

Hechter and Lester (1960) have reviewed the effects of adrenocorticotrophic hormone (ACTH) on different membranes, and find that the movement of both ions and organic substances is affected in the presence of the hormone. Szego and Roberts (1953) and Bitman, Cecil, Hawk and Sykes (1959) found that treatment of rat uteri with oestrogens altered the water and electrolyte composition of the constituent cells. Rasmussen (1959) has demonstrated that parathyroid hormone (PTH) stimulates the transport of calcium across the rat intestine, whilst parathyroidectomy decreases calcium movement. Levinsky and Davidson (1957) also demonstrated that PTH increases urinary excretion of phosphate in the chicken, and a similar effect has been observed in other species. Thyrotrophic hormone (TSH) affects the membrane resistance of the frog and tadpole thyroid follicle (Gorbman and Ueda, 1963), which is indicative of increased ionic movement across the

membrane, and Solomon (1961) has shown that TSH stimulates sodium and water entry into the follicles of chick thyroid. A similar response to water entry has been noted by Bakke, Heideman, Lawrence and Wiberg (1957) in beef thyroid slices treated with TSH.

Although much research has been concentrated upon the effects of circulatory hormones, some effects of more local hormones upon permeability have been noted. The administration of epinephrine or norepinephrine increases the permeability of the cat or dog salivary gland to many molecules having a small molecular radius (Martin and Burgen, 1962). In addition, Majno and Palade (1961) have demonstrated that histamine and serotonin increase vascular permeability, and localized the effect to the venous side of a capillary bed (Majno, Palade and Schoefl, 1961). This brief and from exhaustive outline of hormonal effects on permeability illustrates that more evidence is accumulating of hormones with diverse chemical structure acting by regulating the permeability of cells and cell membranes. Hechter, in his stimulating review (1960), however, has suggested that the apparent differences in permeability of cell membranes, particularly with respect to ions, may not be due so much to a selective transport as to differences in the internal structure of the cell. Hormonal effects on permeability to sodium and potassium, for example, may be exerted throughout the cell, rather than at the cell surface in a similar manner to that of certain crystals which concentrate ions selectively due to their better ability to fit into the crystal lattice. The analogy between the behaviour of crystals and that of the interior of cells may be a larger one than many people are willing to accept

but the suggestion is certainly both novel and stimulating. In addition, Karlson (1963) has presented some evidence that hormonal action may be at the nuclear level, controlling synthesis of enzymes and replicating proteins during development. It is not suggested, however, that this is the only possible mode of action of hormones, but is proposed as a locus of action of developmental hormones, although it may apply to others.

b. History of thyroidal hormones and concepts of their action.

Thyroxine was first isolated by Kendall (1915), and subsequent work by Harington (1926, 1927) identified the compound and determined the chemical structure. Work on identification of constituents of the thyroid gland showed that there was another compound closely related to thyroxine, which could be distinguished on chromatograms. This compound was first identified and analysed by Gross and Pitt-Rivers (1951) who showed it to be 3:5:3' triiodo-L-thyronine, differing from thyroxine in the absence of an iodine atom in the 5' position on the second phenolic ring. More recently there have been various analogues of thyroxine and triiodothyronine either isolated from tissues or synthesised. Thyroxine was shown to alter the metabolic rate of animals, as did triiodothyronine, but thyroxine had a longer latent period of action than triiodothyronine, and this difference led to the concept that triiodothyronine was the 'active' form of the hormone (see Pitt-Rivers and Tata, 1959). The discovery, however, of the fast-acting

acetic acid analogues of the thyroid hormones led these further compounds to be proposed as the 'active' forms (Thibault and Pitt-Rivers, 1955). Much work has been performed *in vivo* on the multitude of analogues now existing, and on their potency compared to the 'mother' hormones (Stasilli, Kroc and Meltzer, 1959; Bruice, Winzler and Kharasch, 1954; Roche, Michel, Truchot and Wolf, 1955). Most of the *in vitro* work has tended to concentrate on the attempt to find the active form of the hormones at the cellular level, such studies, to a large extent, implicating tetraiodothyroacetic acid (TETRAC) and triiodothyroacetic acid (TRIAC). Barker (1962) has reviewed the existing evidence for action of various analogues and of the effects of side-chain substitution and concluded that certain chemical structures, including the occupancy by iodine of the 3' - position, are favourable to hormone action, while that of the 5' - position is not.

The studies of Albright, Larson, Tomita and Lardy (1956) and Tomita, Lardy, Larson and Albright (1957) have shown that heart, kidney and liver of rat contain enzymes which deiodinate thyroxine to yield triiodothyronine, and also that thyroxine and triiodothyronine are converted to their corresponding acetic acid analogues, thus indicating a possible physiological role of these analogues. Etling and Barker (1959) have shown that when rat kidney slices are incubated in thyroxine, the analogues TETRAC and TRIAC are found but no triiodothyronine. Incubation in triiodothyronine produced TRIAC, but added TETRAC did not; it was, therefore, suggested that triiodothyronine is a fleeting intermediate

and rapidly metabolised. The composition of the peripherally-active compound is, however, still in dispute, for despite all the *in vivo* and *in vitro* work which has been performed on the analogues, none of the studies so far reported can account for all the observations seen *in vivo* as a result of thyroxine administration. One of the basic difficulties in analysing these problems has been outlined by Frieden and Westmark (1961) who, after administration of analogues by injection into tadpoles, found no difference between the various analogues as assayed on growth rate. Whereas all the analogues used show differences in potency upon tadpoles immersed in solutions of the hormones when equimolar solutions are compared. This apparent discrepency is at least partly explained by the differences in permeability of the analogues through the epithelial membranes of the animal.

Much work has been performed on the effects of thyroid hormones on enzyme systems both *in vivo* and *in vitro*, but so far none of the proposals has been sufficient to account for the known effects of the hormones. Pitt-Rivers and Tata (1959, pp. 103 to 105) list a whole series of enzymes which have been investigated, but there are no firm proposals to be made for the evidence of thyroid action on any enzyme system from the contradictory and confusing data. The idea that thyroid hormones may influence cellular metabolism by altering enzymes in the cell has preoccupied many workers to the neglect of other fields of investigation (see Hoch, 1962).

Only recently, with the work of Tata (1962), has an attempt been made to investigate the effects of thyroid hormones on whole

intact cells using physiological doses, and, in addition, measuring several parameters such as amino acid incorporation and metabolic effects. Such investigations have revealed that the thyroid hormones may be influencing the permeability of cells to a variety of substrates. The concept of a direct thyroid-enzyme action, in the light of the many inconsistencies in the data, is now strongly doubted, for the variety of enzymes affected by the in vivo administration of thyroxine makes it unlikely that the altered activity of any overall 'pacemaker' will be demonstrated. Some in vitro effects of thyroxine can be explained by an interaction of the hormone with essential metal ions in the medium, but as yet there is no evidence of a direct interaction between thyroxine and a metal-containing enzyme (Tapley and Hatfield, 1962). It may be that this so-called link between the hormone and metal ions has, in fact, little to do with hormonal action, but is simply a requirement of enzymes or enzyme systems for the correct ionic concentration for their catalytic function. The antagonism between magnesium and thyroxine, for instance, can be explained by the effect of magnesium ions in preserving mitochondrial structure (Tapley, 1956). Further, no in vivo alteration of metal metabolism sufficient to explain the effects of the hormone has yet been demonstrated (Tapley, 1962).

Thyroxine has been shown to have a direct effect on mammalian mitochondrial membranes in vitro (Tapley, 1956) and this was demonstrated to occur, also in vitro, at 10^{-8}M, which is a physiological concentration, (Lehninger and Ray, 1957), as well as in vivo, (Schultz, Low, Ernster and Sjostrand, 1956; Paget and Thorp, 1963). This in vivo effect is in dispute, however, for Karlson and Schulz-

Enders (1963), using a more physiological dose, found no swelling of rat liver mitochondria after thyroxine treatment. Mitochondria are known to be the site of generation of the majority of cellular energy and their ability to perform these functions is markedly dependent upon the maintenance of their structure. It may be that the effects of thyroxine, both in vitro and in vivo, are caused by an action of the hormone on the mitochondrial membrane. Recent reviews have summarised the literature on the action of thyroid hormones at the mitochondrial or submitochondrial level (Lehninger, 1960; Tepperman and Tepperman, 1960; Pitt-Rivers and Tata, 1959), but there is increasing evidence to indicate that thyroid hormones can influence processes not directly dependent on mitochondrial function.

Johnson and Redding (1961) worked on mitochondria-free mammalian erythrocytes, and from the results of their work suggest that the metabolic changes induced by thyroid hormones cannot be ascribed to mitochondrial permeability alone. Their findings, however, indicate that the hormones cause a depression of enzyme systems in thyrotoxic patients. Sokoloff and Kaufman (1959) and Sokoloff, Campbell, Francis and Gelboin (1962) as well as Tata, Ernster and Lindberg (1962) have demonstrated that thyroid hormone administration increases protein biosynthesis in various cells. In addition, other extramitochondrial functions such as lipid synthesis and breakdown have been shown to be affected (Myant, 1963). The diversity of action of thyroxine at the cellular level appears to be great, but as yet, no theory of action of the hormone has been able

to unify all these separate effects into one overall governing concept. There is, however, one further factor which controls the supply of substrates and output of metabolic wastes, namely, the cell membrane. Despite the wealth of data which exists on the action of thyroxine on intracellular compartmental membranes, it may be that the hormone in fact exerts its basic control at the overall limiting barrier of the cell surface.

c. Effects of thyroxine on permeability of tissues.

There have been a number of reports of the effect of thyroid hormones on water and electrolyte metabolism in mammals, though many of these are clinical in origin. A number of these are summarised by de Gennes and Bricaire (1951) in a review of thyroid hormone effects in humans and other mammals and they conclude that the hormones appear to cause the elimination of water and the retention of chloride. For example, Brull (1940) found that the basal rate of urine flow from a thyroxine-treated dog kidney was higher than that of a control organ, the water output being approximately seven times greater. The doses used, however, were large and it is possible that these results do not represent physiological conditions. Beisel, Zerzan, Rubini and Blythe (1958) found that a single injection of 1.0 mg. of triiodothyronine into dog caused an increase in urine phosphate excretion as compared with saline-injected controls, the tubular resorption processes falling to approximately seventy percent of the control value. Althausen

(1949) reported that thyroxine injections into dog increased the glucose uptake of the intestine, as well as stimulating fat uptake. In addition, Thompson (1925-26) found that injections of thyroxine into patients caused an increase in blood volume of 25-30%, brought about by a transfer of water from tissues into the blood. Byrom (1933-34), by administration of thyroxine to normal patients, found a loss of sodium and potassium in the following diuretic phase, thyroxine causing a depletion of extracellular water and salts. Here again, however, these effects were brought about only by large doses of thyroxine, for other studies using more physiological doses failed to show any consistent effect on ion excretion (Asper, Selenkow and Plamondon, 1953; Rawson, Rall, Pearson, Robbins, Poppell and West, 1953).

More recently, however, detailed studies by Stephan and his co-workers (1959, a,b; 1960; 1961; 1962) have shown that hypothyroidism in the rat, achieved either by thyroid blocking agents and surgical or radio-thyroidectomy, causes a diminution in renal tubular resorption of both sodium and potassium. A diuretic response was observed which caused a fall in osmolar concentration of the urine. Injection of thyroxine into animals in which the thyroid activity had been blocked resulted in a return of the ionic and water values to normal levels. This work was confirmed by the studies of Fregley, Brimhall, and Galindo (1962), using propylthiouracil-treated rats.

Fontaine (1956) has reviewed the role of the thyroid hormones on water and electrolyte metabolism in fish, and has suggested,

from the confused mass of data, that the hormones, as in mammals, help in retention of chloride and in the elimination of water. Koch and Heuts (1942) studied the influence of orally administered thyroid hormone, and found that the euryhalinity of the stickleback was diminished, thus mineral regulation is upset at normal salinities. The excellent work of Hickman (1959) has placed studies on thyroid control of osmoregulation in a fresh light, for his carefully controlled experiments demonstrated that when fish are transferred to a more saline environment, there is not only an increase in thyroid activity but a concurrent increase in metabolism. This work argues strongly for a calorigenic action of thyroid hormone in at least one species of fish, and if the factors are closely related then it also argues for an effect of thyroid secretions on the membranes concerned with ionic regulation in fish. When faced with an increased salt content of the habitat, fish must respond by absorbing both water and ions, with a preferential excretion elsewhere of the ions. A correlation was drawn between the three increases, namely of thyroid secretion, ionic output and metabolism, which pointed to a direct relationship between the three events.

Few experiments have been performed on thyroid action on cellular permeability in mammalian tissues, but Comsa (1957) has reported that the uptake of glucose by rat diaphragm muscle is increased by 250% in the presence of 10^{-7}M thyroxine. Tapley, Cooper and Lehninger (1955) showed that thyroxine, in physiological concentrations, causes swelling of isolated rat liver mitochondria, and it was suggested by Tapley (1955) that this response is due to a

direct effect of thyroxine on the mitochondrial membrane. During the past year, the work of Tata and others (1963) has demonstrated that the thyroid hormones affect processes such as incorporation of amino acids into proteins in rat liver cells, and that this activity precedes stimulation of metabolic processes. The changes observed resemble in many ways those seen during tissue growth. Their conclusion is that the action of the thyroid hormones may somehow be connected with the interplay between cellular structure and activity. It is becoming increasingly clear that this interplay has a governing role in the control of all cellular processes (Lindberg and Ernster, 1954), and investigations on the effects of hormones on this relationship, as Tata, Ernster, Lindberg, Arrheniu Pedersen, and Hedman (1963) have pointed out, would lead to a far more fundamental knowledge of hormonal action at this level.

d. Anatomy and physiology of toad skin and bladder.

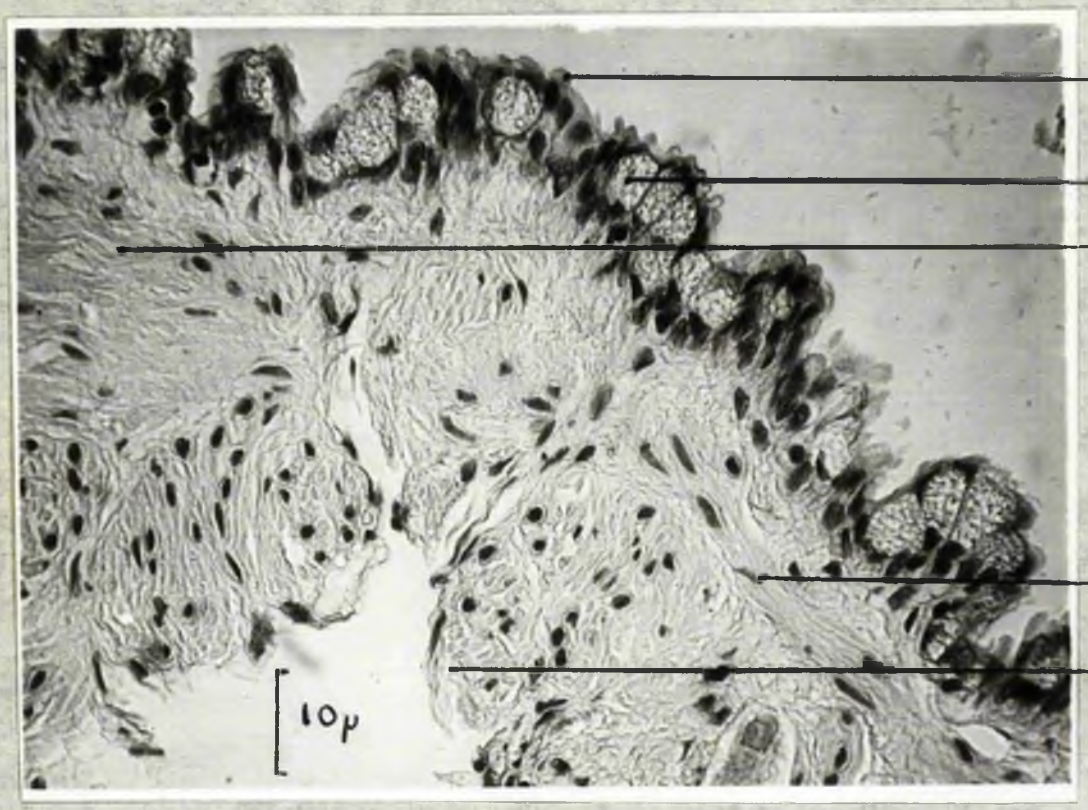
Investigations upon preparations of frog and toad skin and bladder have been used as a basis for the understanding of the movements of salt and water across many biological membranes (Sawyer, 1956). The work of Ussing and Zerahn (1951), Bentley (1958), Leaf (1960 a) and others has shown that the isolated urinary bladder and skin of the Amphibia are particularly suited to the study of hormone action. The membranes are simple in structure, the isolated urinary bladder consisting of a mucosal layer of cuboidal cells and a serosal layer of squamous epithelium, with a

small amount of smooth muscle and a few blood capillaries between them. In the distended state the membrane is roughly 50 to 60 μ thick, and therefore presents an ideal structure with simple compartments. There is also a lack of the numerous secretory glands which are found in the frog and toad skin, and without these complications a clearer picture may be possible, (see Plate I). The skin, although much thicker, is similar in general appearance, although there is a layer of cornified epithelium on the mucosal surface, and an underlying layer of generative tissue, (Plate II).

Work on the intact animal has shown that it can absorb sodium chloride against a very large concentration gradient even when placed in a dilute solution (Krogh, 1939).

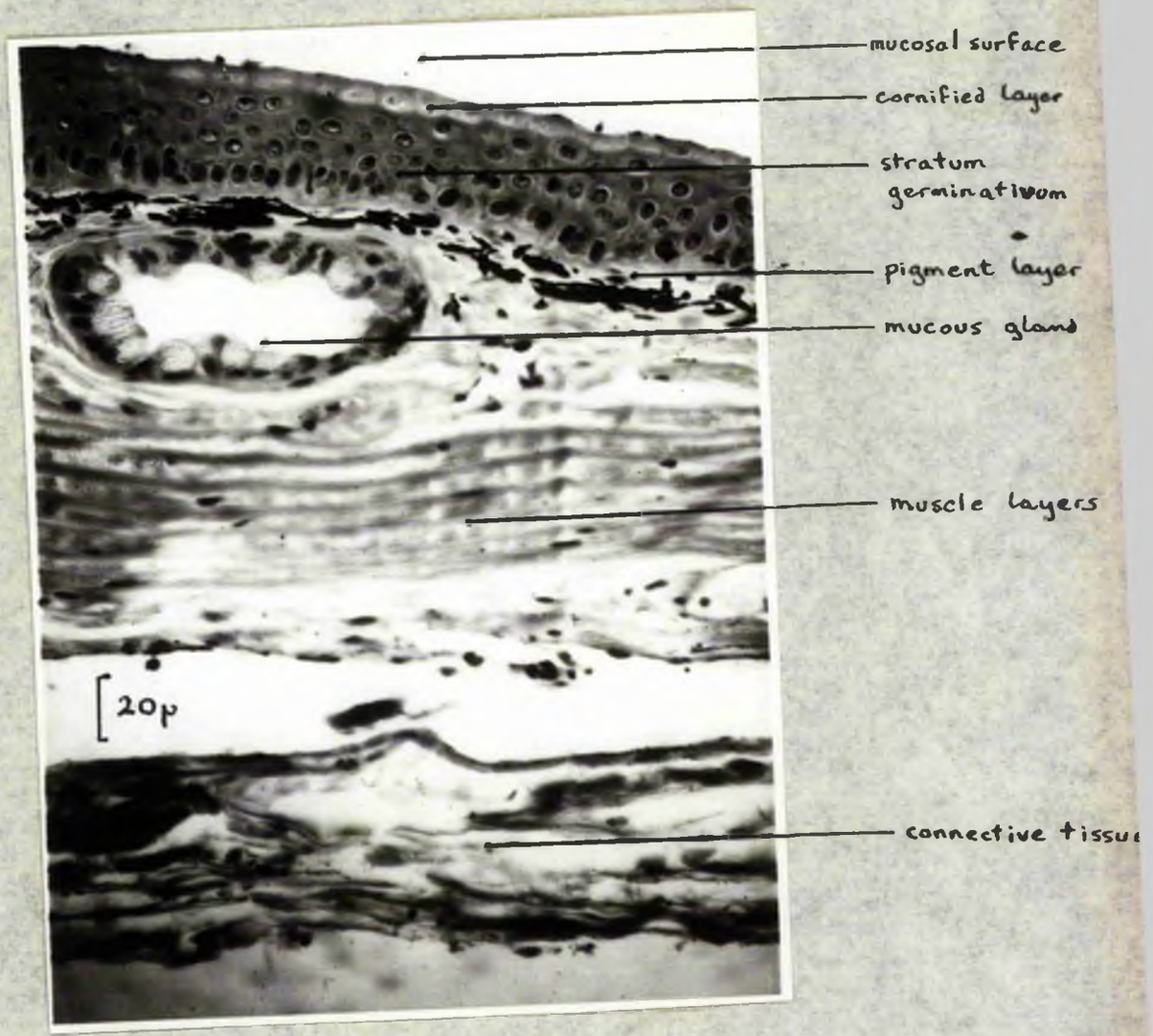
The bladder serves as a reservoir of urine, and resorption of both water and electrolytes occurs into the plasma (Sawyer, 1956). Using these tissues in vitro it is possible to monitor various parameters of activity, all of which are reflections of cellular activity. Both membranes, when removed from the animal and bathed on both sides by similar media, give rise to a potential difference, the serosal surface being positive with respect to the mucosal side. The tissues respire aerobically and in addition exhibit differences in permeability to different substances. Studies by several workers on the biophysics of these membranes have added considerably to our knowledge of hormone action, (Ussing and Zerahn, 1951; Leaf, 1961; see also section a) and similar studies investigating thyroid hormone action were thought to be of possible assistance in understanding the physiological actions in the cell. In

Plate 1



T.S. toad bladder

Plate 11



Toad skin

addition, little is known of the role of the thyroid hormones in adult Amphibia, the thyroid glands of which are known to contain both thyroxine and triiodothyronine (Shellabarger and Brown, 1959), and any work which could add to this confused and somewhat neglected field might be of value.

e. Effect of thyroid hormones on permeability of Amphibian tissue.

Most of the knowledge of the effects of thyroxine in the Amphibia has arisen indirectly as a bi-product of studies on the process of moulting, in which this hormone is known to play a part. Jørgensen (1949) noted that when toads were moulting, both water uptake and sodium intake were enhanced. Koefoed-Johnsen and Ussing (1949), whilst studying the effects of corticotrophic hormone on the ion balance of the axolotl, made a few observations on the effect of injected thyroxine on the salt uptake. Thyroxine increased salt uptake; the effect is quantitatively less than that brought about by ACTH. Levinsky and Sawyer (1952) have shown that thyroxine causes an increase in weight, and hence water content, of cloaca-ligatured Rana pipiens and Heller (1930) also showed that thyroxine had an effect on the water balance of frogs. There have been only one or two reports, however, of the effects of thyroid hormones on isolated Amphibian tissues, including that of Gellhorn and Northrup (1933), where thyroxine in concentrations of 1: 200,000 was shown to increase glucose absorption across Rana esculenta gut. These authors quote the work of Saito (1930),

who found that frog skin showed a greater permeability to dyestuffs both upon addition of thyroid extract to the skin and after feeding frogs with thyroid gland. Previous to this work, Asher (quoted by Loeb, 1923) had performed experiments in which he found that the incorporation of methylene blue into the nictitating membrane of the frog was increased by in vivo thyroxine treatment. In addition, Embden and Adler (1922) reported that thyroid extract, added to the solution bathing frog skin in vitro, increased the rapidity of flow of solution from one side to the other. There is, therefore, a substantial gap in our knowledge of the effects of thyroid hormones both on isolated intact cells and also in the Amphibia.

f. Effects of thyroidal hormones on respiratory metabolism.

Reports of in vivo effects of the thyroid hormones on the oxygen consumption of lower vertebrates are contradictory, although the effects in mammals are well documented (see Pitt-Rivers and Tata, 1959). In teleosts, for example, both Drexler and Issekutz (1935) and Root and Etkin (1937) could find no increase in oxygen uptake after feeding thyroid extract, whereas increases have been reported by Smith and Matthews (1948) and Muller (1953), also in teleosts. The latter author obtained very rapid increases of oxygen uptake after thyroid or thyrotrophin injections. Although Gayda (1922) found no increase in respiration of the toad after

thyroid feeding, a positive response has been obtained in different species of toad by Donoso and Trivelloni (1958) after thyroid hormone injection. Barch (1953) found that implantation of thyroxine pellets also caused an increase in oxygen uptake in the skin of tadpoles or adult frogs.

The reports on the metabolic effects of *in vitro* addition of the thyroid hormones to tissues are few, and those which do exist show discrepancies. Radsma, Golterman and Birkenhager (1954) working on dilute rat liver homogenates, Hoover and Turner (1954) using rat mammary gland, Weiss (1956) on a variety of tissues from different vertebrate classes and Dickens and Salmoney (1956) on rat liver are but four of the many authors who have found no increase in oxygen uptake under the influence of thyroxine *in vitro*. In contrast, Mansfeld (1935) and Yoshihiro (1956) on mammalian tissue, Jacob (1962) using rat liver slices, and Scott (1935) using reptilian tissue have all demonstrated that *in vitro* application of thyroxine to tissues causes an increased oxygen consumption.

Certain of the thyroid analogues have been shown to have immediate effects on different tissues. Recently, Hamolsky, Michel, Carniceroand Roche (1963) reported that triiodothyroacetic acid produced an immediate effect on respiration of horse leucocytes, a response similar to that seen by Bisset and Murray (1962) in human leucocytes. That tetraiodothyroacetic acid has an effect on respiration of tissues has been shown by Jacob (1962) on rat liver and Thibault and Pitt-Rivers (1955) on rat kidney.

In the Amphibia, however, there are very few reports of *in*

vitro thyroxine effects, although both Ahlgren (1925) and Haarmann (1936) observed an increase in oxygen uptake in the isolated skeletal muscle of the frog upon immersion in thyroxine solution. Results obtained from measurements of respiration after injection of thyroxine into the animal, do, however, dispute each other. Warren (1940) found an increase in respiration of frog, whereas Galton and Ingbar (1962 a) found no alteration in frog tissue slice oxygen uptake. The picture is therefore very uncertain and although no single study can hope to provide an answer to the problem, metabolic effects of the hormone were noticed in the present experiments.

g. Present work.

As Sickevitz (1959, 1961) has shown, biological structure and function are closely interrelated, and Hechter (1955) has already suggested that an important function of endocrine secretion may be to regulate this relationship. The need is great, therefore, for a study of the effects of thyroid hormones on cellular activity, particularly on permeability effects, as it is becoming more apparent from present work that control of permeability of both cells and membranes is affected by hormones. In this control of such a fundamental process we may see the locus of action of several different hormones, which provides an explanation of the multitude of other side-effects which are observed as a result of the presence of the hormone.

In the urinary bladder and skin of Amphibia we have, apparently, an ideal system for such studies, particularly as thyroid effects in Amphibia are poorly documented. In addition, these membranes have been used as models of cancerous cells (Ussing, 1960 a) and as analogues to kidney tubules (Leaf, 1960 b), the principles involved in transport processes appearing to be very similar in all these tissues. If these analogies are valid then any work performed on the Amphibian membranes may aid in understanding some of the reports of the thyroid hormone effects on other membranes. The present work, therefore, has been an investigation of the effects of the thyroid hormones on the water and electrolyte permeability of the bladder and skin of the toad as well as a concurrent study on metabolic effects. Further, an attempt has been made to relate findings in vitro to observations made on the effect of thyroxine injections into the whole animal.

SECTION II

The effect of thyroidal hormones on the water permeability of the isolated toad bladder.

A. INTRODUCTION

Robinson (1954) has revieved the subject of active water transport and concluded that there were indications from the experimental data, obtained in various groups of animals, which implied that active water transport was the only explanation for some of the results. Experiments performed on tissue swelling in sodium-free solutions, for example, suggested that the existence of a sodium pump alone could not account for the osmotic behaviour of the cells in tissue slices in these media, and that there exists a water pump to provide independent control of cell volume. Such transport would be dependent upon metabolic energy derived from the cell and not necessarily arising as a result of active sodium, or other cati movement. The large amount of work which has been performed on the analysis of water movement across a number of tissues has, however, produced evidence in the majority of cases examined, that it is indeed a passive process, governed only by physical and chemical gradients. The apparent 'active' movement of water is, in fact, a reflection of active ionic transport which occurs across virtually every system where there is water movement and it is this active ionic movement which creates a driving force for the water shift. This driving force is created either by establishing an osmotic gradient of sufficient magnitude to create water flow, or by drag on the solvent, both caused by the movement of ions through the

membrane. This explanation also accounts for the dependence of water movement upon cellular metabolism since, by causing inhibitic of ionic transport by some means, water movement is also inhibited.

In frog skin there have been few attempts to separate the fluxes of solute and solvent, for the majority of observations have been made indirectly during the course of other studies. Kirschne MaxWell and Fleming (1960), Capraro and Marro (1963) and Koefoed-Johnsen and Ussing (1953), however, have all undertaken studies on water transport across frog skin. The report of Kirschner, where the experiments were performed under a variety of experimental conditions, revealed that water movement can occur in the absence of solute transport. Thus, with no external gradient existing and the resulting flux of water being dependent upon metabolic energy, the requisites for active transport appear to be satisfied. There are, however, several points against the theory and the majority of these arise from difficulties in the experimental technique of separating the two fluxes. The conclusion reached is that althoug water movement may, under certain conditions, be linked to sodium transport this is not necessarily true in all cases. Capraro and Marro reached a similar conclusion after related studies of frog skin. That is, that net passage of water can normally be explained as a result of an osmotic driving force created by ion movement, but under certain conditions net water transfer and active sodium transport can be dissociated and an apparent active water movement occurs.

Hevesy, Hofer and Krogh (1935) measured the water permeability of the skin of whole frogs by immersing them in solutions of differ ent osmotic strength. Simultaneously they measured the diffusion of heavy water into the animals and showed that the experimental re sults were not in agreement with the theory that water uptake was dependent upon osmotic gradients. Following this work, Koefoed-Johnsen and Ussing measured the diffusion of water through isolated pieces of frog skin by isotopic techniques and concluded from their work that the evidence for completely passive water movement was insecure. They demonstrated that water uptake may take place even when the mucosal surface of the skin was bathed with distilled water, a condition where there is a resulting absence of sodium transport. They suggest that when sodium transport does occur thi causes a 'drag' effect on solvent through the pores. The results obtained, as did Kirschner's, allowed the possibility that active water transport may contribute to the transfer process. On the other hand, from their work on frog skin treated with a variety of substances, Schoffeniels and Tercafs (1962) suggest that the passiv movement of sodium and the flux of water are dissociated and take place through spacially separate structures. Results which tend to favour the view of independence of transfer of solute and solven across the membrane, but yield no support for an active water process; water shift appearing to be dependent on osmotic gradients alone.

Earlier studies by Smyth and Taylor (1954) and Fisher (1955) found that water absorption by <u>in vitro</u> preparations of rat small

intestine was inhibited by the absence of glucose in the mucosal solution, and by a variety of metabolic poisons. Later, Parsons and Wingate (1961) found that an *in vitro* preparation of rat intes ine can transport water from mucosa to serosa even though the solu concentration in the mucosal bathing solution is greater than that in the serosal solution. These results have led them to suggest that water transport may be, at least in part, due to some active process. Curran and Solomon (1957) working on rat ileum, *in vivo* and Curran (1960) *in vitro*, have, on the other hand, shown that th dependence of water transport on metabolism is a reflection of its further dependence on active salt transport. This conclusion res upon the fact that water movement and solute movement are linearly related, for the amount of water passing across the ileum wall is proportional to the existing osmotic gradient. Curran and MacIntosh (1962) later performed experiments on a model system whi was devised and under certain conditions an apparent active water movement occurred, but this can be readily explained by considerin the osmotic forces which exist between the cell and the two bathin fluids. The experiments illustrate that the model proposed by Curran (1960) could serve as a model for water movement by the in- testine. They also lend further support to the conclusion that intestinal water movement is a passive process, although it is closely linked with active solute transport. Such conclusions are in agreement with the experimental work of McDougall and Verzar (1935), Rossi and Capraro (1961) and that of Green, Seshadri and Matty (1962). The latter authors, by applying an osmotic gradient

from serosal to mucosal surfaces (mucosal fluid - hypertonic), stopped water movement without affecting the solute transfer, thus indicating the passive nature of the water shift and the independence of the two processes under these conditions. Windhager, Whittembury, Oken, Schatzmann and Solomon (1959), Whittembury, Oken, Windhager and Solomon (1959), and Windhager and Giebisch (1961) have shown, in Necturus and rat kidney tubules respectively, water movement to be dependent only upon the osmotic concentration on either side of the membrane. The net water flux may be accounted for quantitively in terms of the osmotically induced forces arising from net solute movement and net water flux was zero when net solute uptake halted.

Leaf and Hays (1961, 1962) have ably demonstrated that water movement across the isolated urinary bladder of the toad is passive. They showed that the unidirectional permeability to water, measured isotopically, was large in the presence of, or absence of an osmotic gradient. In the absence of an osmotic gradient the permeability to water was found to be equal in both directions across the membrane and the values obtained indicate no net movement of water. Gravimetric determinations of net water flux are within the limits of the technique employed and again indicate equal permeability in both directions. Even with large osmotic gradients there occur only small, but readily measurable, net transfers of water. The finding that net water movement is proportional to the transmembrane osmotic gradient and that no water movement occurs in the absence of such a force, indicates that water moves passively

across the membrane(Hays and Leaf, 1962a). This data is consistent with the *in vivo* finding that dilute urine remains in the bladder for long periods of time with little or no detectable difference in concentration. It was also shown that active sodium transport by the membrane contributes very little to the net water movement. This was shown in two conditions where sodium transport was abolished. Firstly, by replacing all the sodium in the medium with choline and secondly, by removing all the potassium from the serosal surface fluid, which again stops active sodium transport. In both conditions the same relationship of water movement with osmotic gradient still holds.

Various hormones, including aginine vasotocin, arginine vasopressin, lysine vasotocin, lysine oxytocin, oxytocin (Sawyer, 1960, 1961a,b; Jard, Maetz and Morel, 1960), and histamine dihydrochloride (Green and Matty, unpublished) have been shown to increase transfer of water down an osmotic gradient across the isolated toad or frog bladder. The recent work of Rasmussen, Schwartz, Young and Marc-Aurele (1963) has extended previous observations on the neurohypophyseal hormone analogues and investigated the relative potencies in relation to the chemical structure of these substances. These studies indicate that certain chemical requirements are necessary for the hormone to bring about its characteristic response. Several other hormones have been shown to effect the permeability to water of various tissues and membranes, thyrotrophic hormone, for instance, increases the water content of the chick thyroid (Solomon, 1961) and of ox thyroid slices (Bakke,

Heideman, Lawrence and Wiberg, 1957). Oestrogen and progesterone, when injected into female rats, cause uptake of water into differen cellular compartments within the uterine tissue (Szego and Roberts, 1953). Much work has been performed on the anti-diuretic effect of the pressor principles of the neurohypophysis and their action on kidney resorption (see Heller, 1950). While Chester Jones (1957), in his book on the adrenal cortex, illustrates that the adrenal steroids exert a control of water balance in various groups of animals and also control the water content of tissues.

It has been known for many years that thyroxine causes diuresi in hyperthyroid patients and when administered in high doses to mammals (see Pitt-Rivers and Tata, 1959), but few carefully controlled experiments have been performed to investigate this effect. Recently, however, Stephan and his colleagues (1960, 1961) have shown that in hypothyroid rats water resorption by the kidney tubules is decreased and this was confirmed by Fregley, Brimhall and Galindo(1962). Both groups obtained results which indicate a decrease in resorptive capacity of the kidney tubules, both of ions and water during thyroid insufficiency.

Such studies have added considerably to our knowledge of the permeability of membranes and of hormone action. No in vitro studies, however, have been made on water permeability effects of the thyroid hormones on any membranes, save that of Embden and Adler (1922) who found that frog skin showed an increased flow of solution between the bathing media after thyroid treatment in vitr It seemed likely that the isolated toad bladder could be used to

investigate the effects of these hormones on water transport and further, to examine thyroidal hormone effects on membrane behavio

Leaf (1955, 1960a) and Leaf, Anderson and Page (1958) have shown by both electrical and isotopic measurements that the bladders of Bufo bufo and B. marinus transport sodium actively. The relationships between sodium transport and water movement have been outlined above and this relationship between water movement and sodium transport has been shown in addition to be allied to cellular metabolism. Leaf and Renshaw (1957) and Zerahn (1956), using frog skin and Leaf, Page and Anderson (1959) using Bufo marinus bladder, were able to show that there exists a close relationship between oxygen molecules consumed and sodium ions transported. Furthermore, this relationship holds during periods of increased or decreased metabolism of the tissue. To study these relationships and investigate the action of thyroxine under impaired metabolic conditions specific inhibitors of certain enzymes concerned in cellular metabolic processes were used, as well as general inhibitors of metabolism. In order to investigate the effects of sodium depletion on the water movement response elicite by the thyroid hormones, experiments were performed using a sodium free saline. Such studies would reveal how the thyroid hormones were acting on the membrane and assist in the understanding of their action.

As previously stated, in the absence of an osmotic gradient there is little net water movement across the isolated toad bladde but although this is slight it is generally found to be from the

mucosal to serosal surfaces - in the direction of active sodium transport. Since investigations revealed that the thyroid hormones caused an increase in water movement down an osmotic gradien studies were made on the effect of the hormones on these small water movements.

Whereas insulin, vasopressin and other hormones are thought to be the actual substances that elicit the characteristic respons in different tissues, such direct action of thyroxine and triiodothyronine is a matter of controversy. Although the hormones are known to be released from the thyroid without alteration and have been detected in the blood (Taurog and Chaikoff, 1948; Taurog, Wheat and Chaikoff, 1956), it has been suggested that the thyroid hormones may be converted into metabolites such as the acetic acid analogues, tetraiodothyroacetic acid (TETRAC) and triiodothyroacetic acid (TRIAC), which are the active forms of the hormones. This concept of thyroidal hormone action was introduced by the finding of Pitt-Rivers (1953) that the acetic acid analogues of triiodothyronine and thyroxine had definite biological activity. Studies <u>in vitro</u> by Thibault and Pitt-Rivers (1955) have also shown that these analogues have an immediate effect upon biological oxidative processes, both TRIAC and TETRAC producing their maximal effect on oxygen uptake of rat kidney slices after fifteen minutes. These effects were confirmed <u>in vivo</u> by Thibault (1956) working on adult rats. This work, however, was not confirmed by the studies of Wiswell and Asper (1958) and of Barker and Lewis (1956). Further confusion was added by the isolation by Galton and Pitt-

Rivers (1959) of TETRAC and TRIAC from the liver and kidney of th mouse, providing more evidence of the possibility of these analog being the active principles of the thyroid hormones.

Pitt-Rivers and Tata (1959) have listed some objections to the idea that these analogues are in fact the 'active' forms of th hormones. Firstly, the latent periods of triiodothyronine and TRIAC are the same in myxoedematous subjects; secondly, that a rapid conversion of thyroxine and triiodothyronine to the acetic acid analogues is seen both <u>in vivo</u> and <u>in vitro</u> and the time tak for this conversion is shorter than is the latent period of actio of either of the parent hormones. They also critisize the use of analogues <u>in vitro</u> when added to the bathing medium and also when injected into the animal, on the grounds that this represents an 'unnatural' condition as substances must pass into the cell, a process which is not thought to occur during the intracellular de-iodination or degradation of the hormones. Although transfer inwards may be unnatural, if a substance such as TRIAC or TETRAC has been shown to be present within the cell, then there is no reason to suppose that the <u>action</u> of the substance once inside the cell will differ in its <u>effect</u> from the same substance of cellular origin. Until more knowledge of thyroid hormone metabolism and the production of analogues becomes available, further speculation is unnecessary. Experiments were, however, performed using certain analogues on the isolated toad bladder membrane, to compar their speed of action and quantitiative effects with those of the parent amino acids.

As mentioned above, studies by Etling and Barker (1959) on incubation of kidney slices in thyroxine led them to suggest that triiodothyronine was a fleeting intermediate in the degradation of both thyroxine and of TETRAC. With this in mind experiments were performed using triiodothyronine, the results of which implied that it was rapidly metabolised and further experiments were performed to investigate this phenomenon.

An attempt to elucidate the action of thyroxine and triiodothyronine and their analogues on water permeability at the membran level is presented.

B. MATERIALS AND METHODS

1. Animals

Mature Bufo bufo of both sexes were used throughout this investigation. They were obtained from a commercial source (L. Hall & Son, Surrey) and immediately upon arrival placed in a constant temperature room at 12°C. The animals were not fed during the period prior to the experiment since they arrived in small numbers at regular intervals, appearing to be very healthy upon arrival and remaining so during the short investigational period. They were allowed to acclimatize for two days and shortly before use were allowed to come to ambient temperature (17-20°C). Animals were not kept for longer than fourteen days after arrival.

2. Saline solutions

The saline solution used had the following composition:-

NaCl 6.5 g./l., KCl 0.2 g./l., $CaCl_2$ 0.2 g./l., $MgSO_4$ 0.1 g./l., $NaHCO_3$ 1.5 g./l., Na_2HPO_4 0.1 g./l., and glucose 0.75 g./l. The pH was normally 7.8, but was always adjusted to this value by the appropriate KOH or HCl solutions. The osmolarity of the saline was 260 milliosmoles. In experiments where an osmotic gradient was required, the above saline was lowered to 20% of its original strength by the method of dilution. The remaining dilute solution had an osmotic activity of 60 milliosmoles. Fresh saline, both normal and diluted, were made up every three days, or as required and stored in stoppered flasks at room temperature.

3. Preparation of isolated bladder.

The pithed animal was pinned out on a cork board and a median abdominal incision made, which cut the skin and underlying muscle to expose the contents of the abdomen. The bladder is a transparent, bilobed structure sometimes filling one third of the body cavity. It was found that less physical damage was done to the membrane, when it was fully distended, by cutting it slightly at the base and thus allowed the urine to run out. The contracted bladder was then removed by lifting each lobe separately with a pair of blunt-ended forceps and cutting away the loose mesentery with which it was attached to the body wall. The free bladder was cut out of the animal and placed in a petri dish containing saline at 25°C. The two halves of the bilobed structure were cut apart and, using fine-ended but dulled forceps, the half bladder was opened at the neck which is formed when it is separated from the other half. This single lobe was placed over the end of a

Pyrex glass tube, external diameter $\frac{1}{8}$", and tied on. The free end of a piece of capillary polythene tubing, attached to a syringe, was inserted down the tube and into the spherical bladde The solution in the bladder was withdrawn and replaced, using a different syringe and attachment, by a saline solution diluted to 20% (v/v) or normal, undiluted, saline solution at 25 ± 0.5°C. Between 100 - 400 µl were required to fill the bladder. The bladder was then suspended inside a test tube containing 15 ml. of undiluted saline. Before the bladder was tied on, the glass tube was pushed through a rubber bung which fitted the test tube and supported the bladder in the saline solution. All solutions were at 25°C. before being applied to the bladders. The techniq follows that devised by Bentley (1958).

4. General procedure.

The experimental procedure closely followed that of Bentley (1958), except for a slight modification of times. The procedur was as follows:- after removal from the animal and when the sali inside the bladder had been replaced, the half bladders were allowed to equilibrate for forty-five minutes in the saline solution. The outer solution was continually aerated, the air passi through fine polythene tubes which were sealed at one end and pierced a number of times with a pin. It was originally found that if the aeration was too vigorous the membrane was damaged and the preparation had to be discarded. Air was supplied from a small aquarium pump.

After the forty-five minute equilibration period, the bladder

was emptied using one syringe and tube attachment, rinsed once and refilled with fresh solution via the other syringe. The bladder, glass tube and attached rubber bung were then weighed and suspended in fresh, aerated saline so that the top of the bladder was just covered.

After a forty-five minute control period the bladder and attachments were weighed again, any weight loss being taken as a measure of fluid lost by transfer to the outside solution. The fluids were changed both inside and out and the bladder immediately re-weighed. It was then returned to the fresh, aerated saline, but in this instance hormone was added to the solution. A further weighing was made after sixty minutes (sometimes after forty-five minutes) incubation in the saline-hormone solution. The saline solution was then removed, the bladder rinsed and new solution introduced, the bladder then being allowed to wash for a further f forty-five minutes in fresh normal saline. Finally the inside and outside solutions were changed once more and a second control period of forty-five minutes performed. Sometimes this was followed immediately by a second treated period, a forty-five minute washing period and a third control period. The bladder was finally punctured and allowed to drain completely before re-weighing. In some experiments weighings were made at ten minute intervals during the first control and the treated periods. No experiments were continued for longer than six hours and if the second and third determinations of control water loss were clearly different from the first one the set of results was discarded.

The changes in water loss when expressed graphically are presented as increase over the normal untreated values obtained from the first and second control periods.

5. Precautions

It was found that the bladders normally retained their spherical shape, although sometimes they underwent spontaneous contraction, forcing their fluid contents into the glass tube. These contractions, however, usually lasted for under a minute and showed no effect on the data. When filling the bladder it is important that there should be no hydrostatic pressur difference between the two media and the arrangement used was as follows:- a test-tube was allocated to each glass tube and these were kept as permanent pairs throughout the course of the experiments. Ont each vessel was cut a mark indicating the level at which the bladder was tied onto the partnering glass tube. This enabled the test-tube to be filled to the same level for each experiment (i.e. 15 ml.) in which it was used, that is approximately half way up the tube, and by filling the bladder to the level of the ligature consistency of hydrostatic pressure was maintained. Althoug since the bladder was filled before immersion in the bathing medium a slight amount of fluid sometimes rose into the glass tube when the bladder entered the bathing fluid. When this occurred after the initial filling, the fluid in the tube was immediately removed and the bladder re-weighed. Care was taken to refill the bladder so that each succeeding weight was within 2% of the initial weight.

The technique is simple and effective but the precautions mentioned must be observed as should those concerning the weighing procedure.

Differences in the mode of withdrawal of the glass tube prior to weighing the bladder can lead to discrepancies in accuracy of weighing and a standard procedure must be adopted to remove adsorbent water. The whole of the tube was kept dry above the leve of the fluid by regulations of aeration. Aeration should be kept at a low rate, since vigorous bubbling of air past the bladder causes damage to the tissue as well as wetting the glass tube. The glass tube was supported in the test-tube by means of a rubber bung and care should be taken that this also is kept dry. It was found preferable to withdraw the bladder straight from the bathing fluid and to prevent contact with the sides of the test-tube. Th bladder was allowed to touch the inside surface of the test-tube a about two thirds of the way to the top and it was then wiped three times on the surface of the glass in a circular motion before being lifted cleanly from the tube. The bladder and glass tube were then weighed immediately before evaporation from the surface of the tissue could cause unwanted errors in the weight.

6. Theory of technique and expression of results.

To facilitate comparison with work on frog skin the area of the bladder was calculated from its volume, assuming that the filled bladder was spherical (Sawyer and Schisgall, 1956). The mean of the initial and final volumes from each experiment was used and this together with the weight of the empty bladder at the

end of the experiment enabled the volume to be calculated. From this value the radius of the sphere can be calculated and from the radius a value for the area of the membrane is readily obtained using the well-known relationships, volume = $\frac{4}{3}\pi r^3$ and area = $4\pi r^2$. This method is not precise but affords a calculation which is sufficient for comparison with other work. It was also assumed that the fluid moved has the same density as that in the bladder, but this may not be so, since there is a known active ion movement from mucosal to serosal surfaces. In these experiments, however, such differences are slight and can be safely neglected.

When the area is calculated as above and one mg. of fluid is assumed to equal one µl. of fluid, the data can readily be obtained in the requisite terms.

The size of the bladder governs the amount of water loss, assuming the loss per unit area to be the same, as is shown in Fig. 1. It can easily be seen that 100mg. loss of weight from a bladder 1 ml. in size corresponds to 2 µl per unit area, whereas, the same weight loss from a bladder on only 250 µl. corresponds to a water loss of 7 µl. per unit area (see also Table 1).

7. Balance

A stanton C L1, single pan, semi-automatic balance was used for all procedures where weighing was necessary. The weighing time was reduced to ten to eighteen seconds by weighing the glass tubes and bungs prior to the experiment and keeping each one catalogued.

Fig. 1. Theoretical water loss from bladders of different sizes

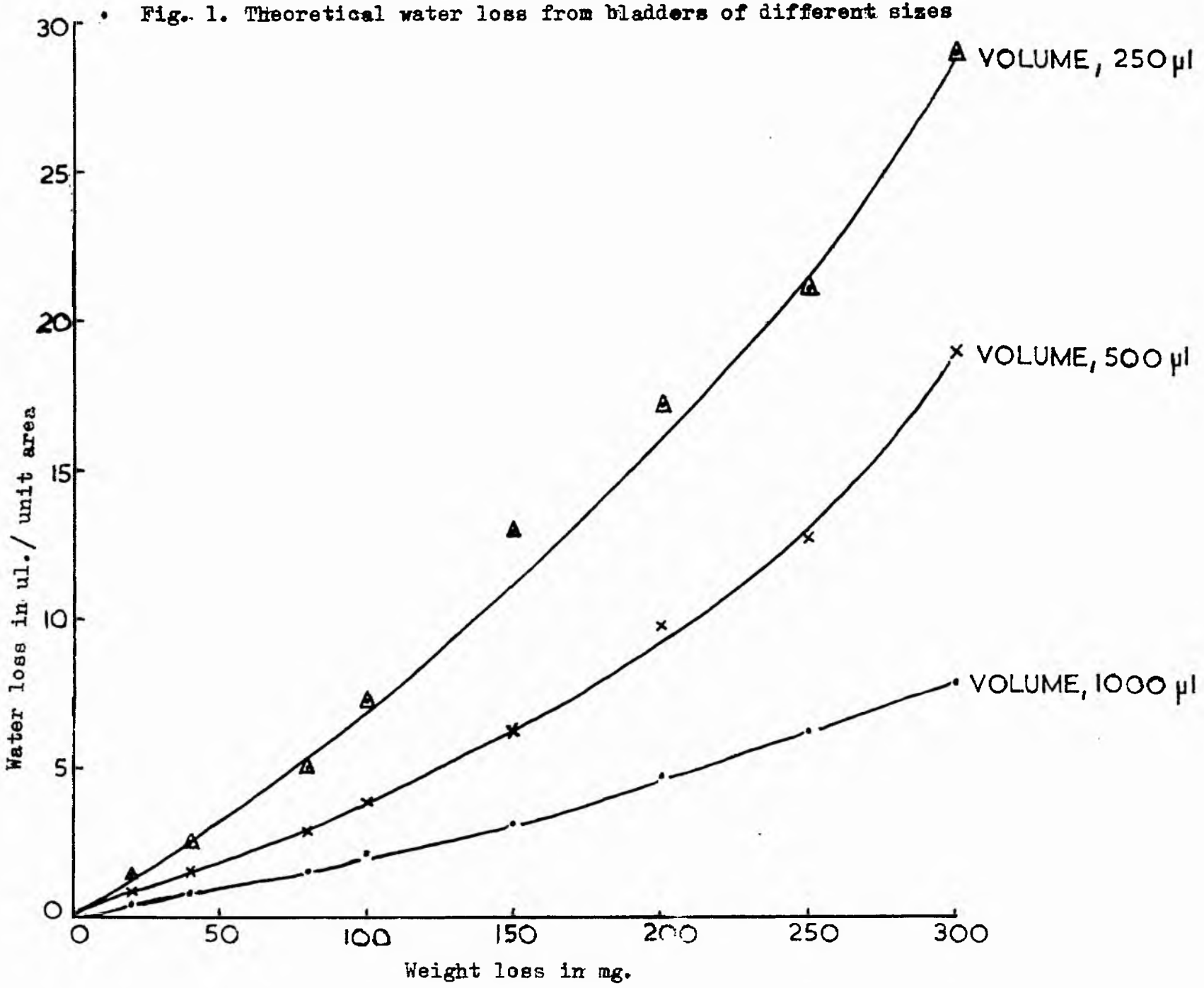


Table 1. Theoretical water loss values from bladders of different sizes.

Weight loss in mg.	Water loss/unit area for different sizes of bladder. 250 ul	500 ul	1000ul
20	1.11	0.65	0.41
40	2.42	1.35	0.83
80	5.00	2.79	1.68
100	7.15	3.80	2.12
200	17.21	9.50	4.74
300	28.80	18.74	7.75

8. Weighing accuracy.

The weighing accuracy was determined for three bladders, with normal saline inside and out, by weighing them nine times at intervals of four minutes. The total weight loss changes were (mean ± S.E.) 0.54 ± 0.15/mg.; 0.82 ± 0.16/mg.; and 0.30 ± 0.05/mg. The individual weight losses per four minutes gave a standard error of ± 0.15 mg. It is therefore, of low significance except, as for Bufo marinus (Bentley, 1958) when measuring small water movements. Results are compiled from the means of different bladders and often the biological variation between one bladder and another is sufficient to make weighing errors of little importance. When half bladders are compared, however, these weighing variances may become important, for it was found that the water loss values from the lobes of a bilobed bladder show very little difference.

9. Everted Bladders

Everted bladders were prepared by turning one half of the bilobed bladder inside out and in each case where this procedure was used the other half bladder was retained in its normal orientation and treated in the normal conditions. Water transport was measured by the usual procedure and was compared with the other lobe. Thus hormone applied to the serosal surface of the normal bladder would be placed on the mucosal surface of the everted bladder by virtue of its eversion.

10. Inhibition

Enzyme inhibitors (Analar grade) were used in concentrations of 10^{-6}M, at which concentration specific enzyme systems have been

shown to be inhibited (Baldwin, 1959). Reduction of temperature to 4.5 ± 0.5°C., using a refrigeration unit attached to a water bath and the perfusion of preparations in a glucose-free saline with nitrogen instead of air were also carried out separately in order to investigate the normal permeability and the effects of thyroxine on the bladder under impaired metabolic conditions. In each case of the addition of a chemical inhibitor it was added to the medium bathing the serosal surface of the bladder; the thyroxine was also added to the serosal surface. In all these experiments one half of the bilobed bladder was used with thyroxin under normal conditions at 25°C. The course of the experiment was as described previously, that is, two control periods with an experimental period between them with a washing phase between the treated and second control periods during which the saline was changed twice. The washing period during these inhibitor experiments was one hour instead of forty-five minutes normally employed.

11. Sodium-free incubation media.

A choline medium was used for some water-loss studies which had the following composition:- choline chloride 8.5 g./l., KCl 0.2 g./l., $CaCl_2$ 0.2 g./l., $MgSO_4$ 0.1 g./l., $KHCO_3$ 1.2 g./l., K_2HPO_4 0.2 g./l., and glucose 0.75 g./l., dissolved in deionised water. The pH was adjusted where necessary with KOH or HCl to 7.8. This medium was stored at 4°C. in 'Pyrex' bottles during use and kept for not more than two days after preparation. A volume of choline medium sufficient for experiments was raised to

25°C. in a water bath immediately prior to the experiments. The lobes of the bilobed bladder were washed for one hour in choline medium which was changed twice during this period. Similarly, with the other half of the bladder, which was treated in sodium medium, the serosal and mucosal bathing fluids were replaced twice during the hour washing period. This was done as a control against handling effects which may have occurred. The same general procedure was followed as described in II,4, thyroxine being applied to the serosal surface only.

12. Isosmotic conditions

Twenty-four half bladders (a,b) of twelve animals were divided as shown in Table 2. A further twelve animals (twenty-four half bladders) were used as Table 2 but in this case the half bladders were everted. Each half bladder was filled with undiluted saline and the water loss measured gravimetrically. Water loss (or gain in the everted condition) was measured after one hour incubation in hormone. Thyroxine was dissolved in the saline bathing either the serosal, mucosal or both surfaces at the same time.

13. Re-incubation in triiodothyronine.

Half bladders filled with 20% (v/v) saline were immersed in triiodothyronine solution in normal saline and weighed every ten minutes for sixty minutes. A freshly washed bladder for which a control water loss had been obtained, was placed into the same incubation medium and this bladder weighed every ten minutes for sixty minutes. Finally a third freshly washed bladder was also

Table 2. Division of bladders for water loss measurements under iso-osmotic conditions.

Thyroxine, mucosal	1a	2a	-	4a	5a	-	7a	8a	-	10a	11a	-	
Thyroxine, serosal	1b	-	3a	4b	-	6a	7b	-	9a	10b	-	12a	
Thyroxine, both sides	-	2b	3b	-	5b	6b	-	8b	9b	-	11b	12b	

incubated in the same medium and further weighings made. It was considered unnecessary to perform a second control period on the bladders, as the water levels were, by the end of their incubation period, at control levels.

14. Thyroxine-triiodothyronine response.

Both hormones were added to the serosal surface of the bladder in normal saline at the same time, at a concentration of 10^{-6}M for each, and water loss was measured every ten minutes for sixty minutes. Each half bladder was filled with 20% (v/v) saline. The normal procedure was followed throughout this experiment.

15. Analogues

3;5;3';5'; tetraiodothyroacetic acid (TETRAC), 3;5:3' triiodothyroacetic acid (TRIAC) (Glaxo Laboratories Ltd.), 3:5:3';5' tetraiodothyroproprionic acid (W 1524, Lot 8) and 3:5:3';5' tetraiodothyroformic acid (" 1489, Lot 00125) (kindly provided by the Warner-Lambert Research Laboratories, Morris Plains, New Jersey) were dissolved in a minimal amount of 0.1 N NaOH before addition to the saline. Similar amounts of NaOH were added to the control media. No hormonal solutions were used more than three days after making up. They were stored in stoppered flasks at room temperature. When measuring water movement induced by analogue treatment weighings were made every two minutes for the first ten minutes instead of taking the first reading after ten minutes. This was necessary, for initial work, when readings were taken at ten minute intervals after the addition of the analogues, showed that the water loss was falling from some unknown

peak at the ten minute reading. Two minute weighings appear to be the limit of the technique originated by Bentley (1958). The procedure followed that of the other hormone treatments.

16. Hormones

L-thyroxine (L. Light & Co. Ltd.) and 3:5:3' triiodo-L-thyronine (Glaxo Laboratories Ltd.) were dissolved in a minimal amount of 0.1 N NaOH except when required in choline media when similar amounts of 0.1 N KOH were used. NaOH or KOH were added to control media. No hormonal solution was used more than three days after making up. They were all stored in stoppered flasks at room temperature, except when in choline medium when they were stored at 4°C. and a suitable amount, as required for the experiment, was raised to 25°C. before addition to the membrane.

C. RESULTS

1. Effects of Thyroxine and Triiodothyronine on water movement.

The control values (mean ± S.E., with number of experiments in parenthesis) for water loss were 11.69 ± 1.05 µl./cm.^{2}/hr. (236) for thyroxine experiment and 11.51 ± 0.88 µl./cm.^{2}/hr. (144) for those with triiodothyronine.

The effects of thyroxine and triiodothyronine in concentrations of 10^{-8}M to 10^{-5}M are shown in Fig. 2. The increases in water movement brought about by thyroxine and triiodothyronine during one hour are of a similar order, both giving a dose response

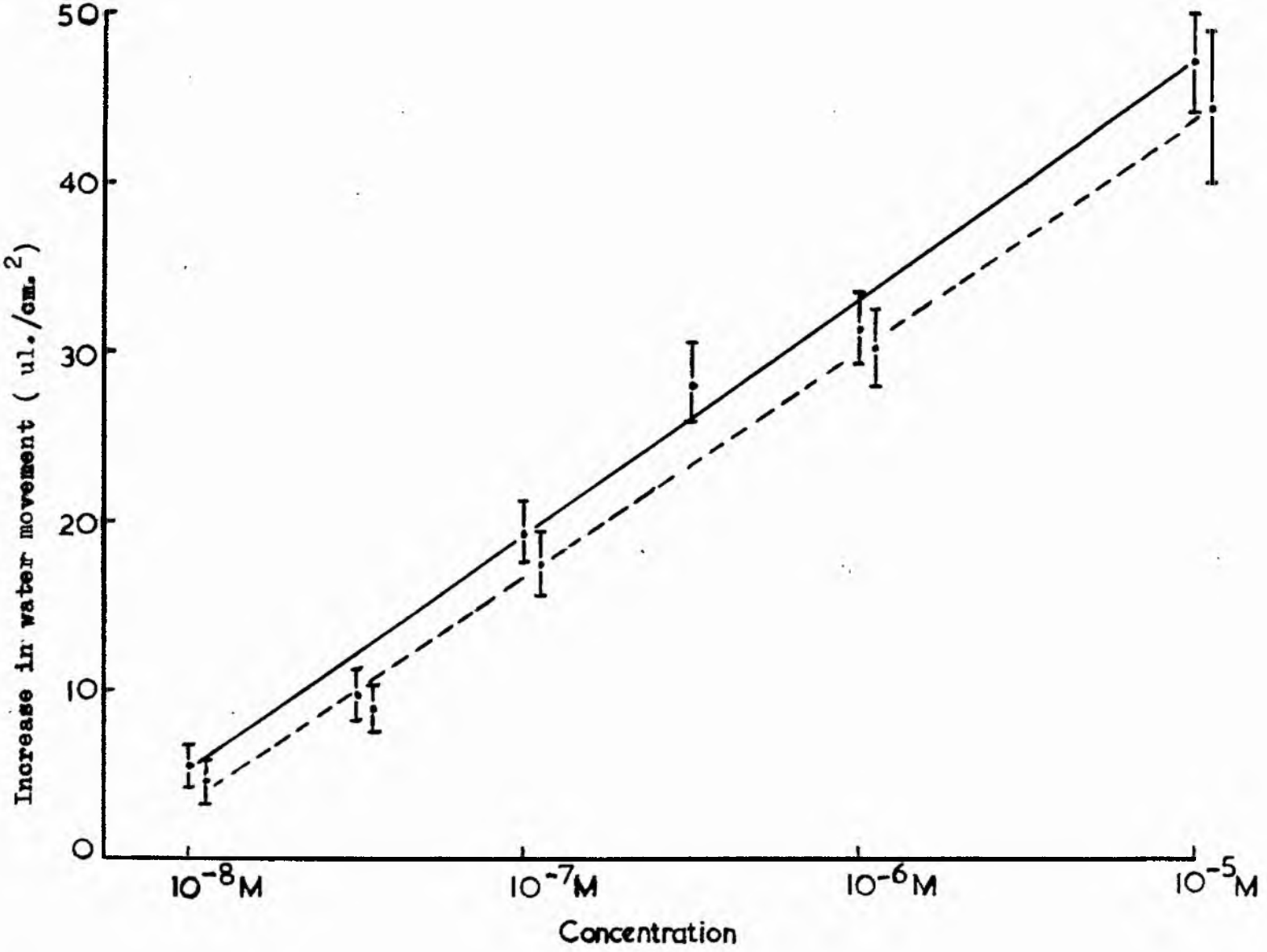


Fig. 2. Effect of varying concentrations of thyroxine (-) and triiodothyronine (- - -) on water movement across isolated toad bladder. Each point represents the mean S.E. of twelve half-bladders.

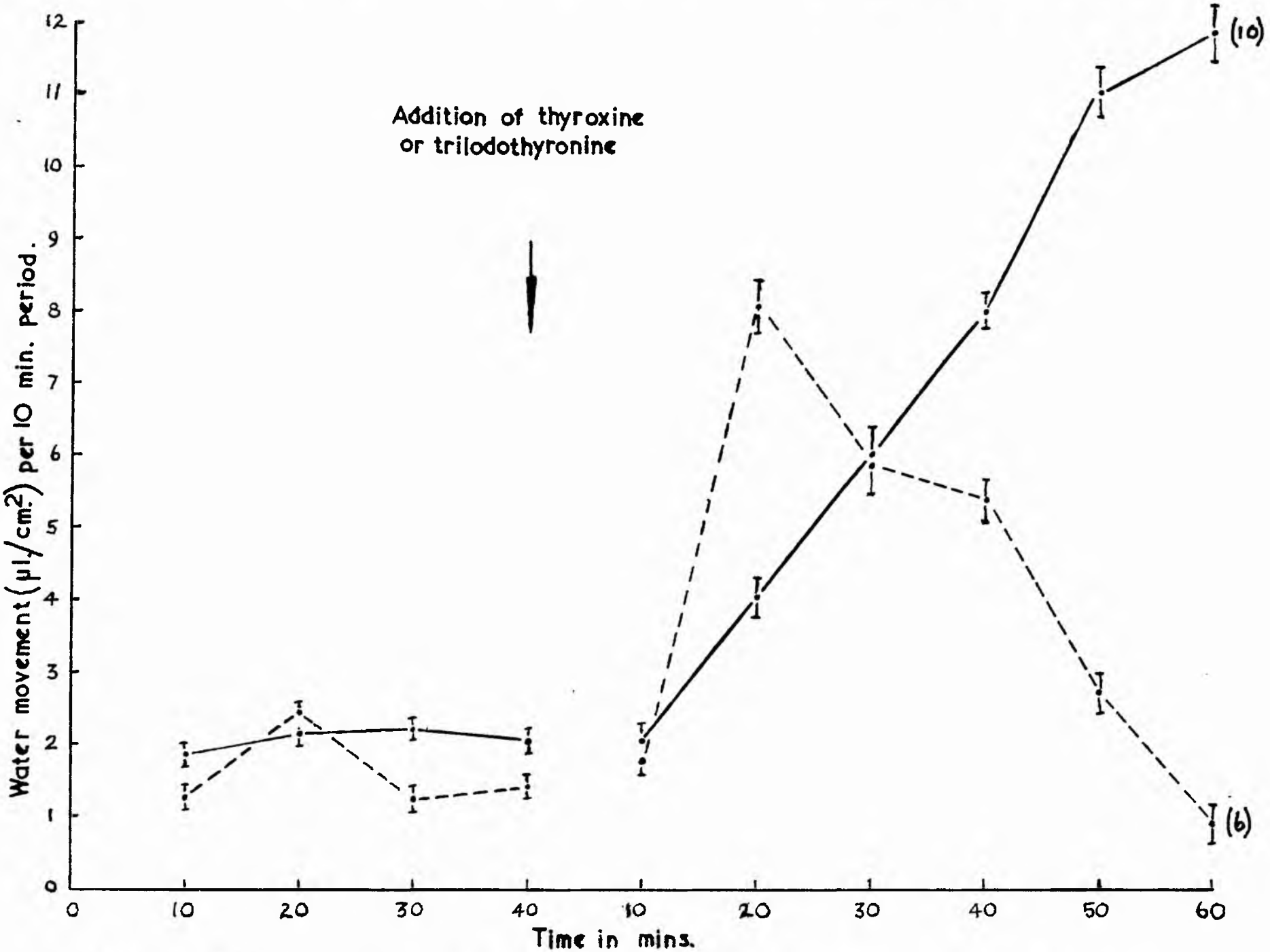


Fig. 3. Effect of 10^{-6} M thyroxine (–) and 10^{-6} M triiodothyronine (– – –) on water transported per 10 min. period across the isolated toad bladder. Note: the water-loss values are not cumulative.

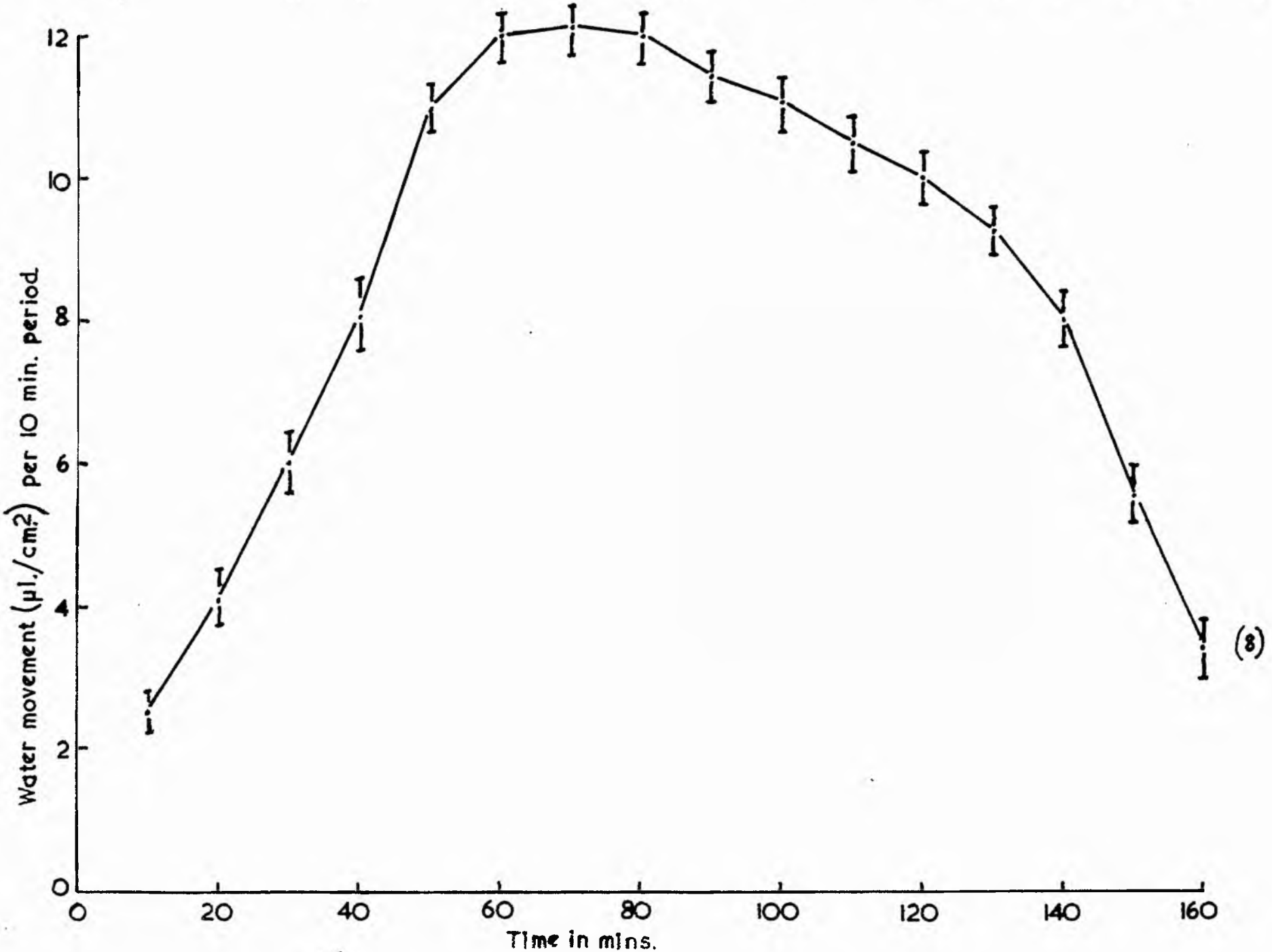


Fig. 4. Effect of 10^{-6} M thyroxine concentration on water loss per 10 min. period from the isolated toad bladder. The water-loss values are not cumulative.

relationship. During 1 hour incubation at 10^{-5}M the water loss is approximately 50 µl./cm^2. which is about x 5 that of the cont period.

With regard to dose response, however, it must be realized that although differing in their time course of action, equimola solutions of triiodothyronine and thyroxine appear to cause the same total movement of fluid during the first hour of incubation this holds only for readings taken at the end of sixty minutes; at other times a linear log dose would still be obtained.

Fig. 3 shows the water losses occurring over ten minute periods before and during incubation in 10^{-6}M thyroxine and 10^{-6} triiodothyronine. Whereas thyroxine induces a steady increase i water loss over one hour, triiodothyronine results in an initial rapid water loss which falls after fifty minutes to control level Continued incubation of a bladder in thyroxine solution was carried out and, as can be seen in Fig. 4, the water loss values, after peaking at sixty minutes, show a gradual fall until after one hundred and thirty minutes incubation the values have returned to control levels.

The effect of thyroxine on water loss from dead bladders was investigated. Four bladders were killed in 4% phenol, the membrane retaining its flexibility without becoming fragile. Mean water losses were 121.2 ± 3.5 and 109.7 ± 3.5 µl./cm^2./hr. in the control periods and 99.2 ± 6.5 µl./cm^2./hr. in the presence of thyroxine. These experiments were performed as a control against any decrease of tissue health which may have taken place during

the experiments. Irreversibly high water loss values were therefore indicative of deterioration of the tissue. It was found that phenol effectively killed the bladders, as did 5% formalin, but with the latter substance the bladders became easily damaged.

The effect of added adenosine triphosphate (ATP) was investigated on the water loss down an osmotic gradient from the bladder, both in the presence and absence of thyroxine. No difference was observed between the water loss values from half bladders treated with ATP alone and the control values, and similarly no statistical difference could be found between the water loss from half bladders treated with thyroxine plus ATP and those treated with thyroxine alone. Control values of 11.73 ± 1.13 (10) became 11.56 ± 1.07 (10) μl./cm^2./hr. after addition of ATP, and values of 39.66 ± 1.62 (10) for thyroxine treatment became 38.92 ± 1.96 (10) μl./cm^2./hr. incubation with thyroxine plus ATP.

2. Everted bladders.

From each of the twelve whole bladders, one half bladder was suspended in the normal condition and the second half in the everted condition. The experiments were performed with an osmotic gradient present in both normal and everted conditions. In each case 20% (v/v) saline was placed on the inside of the sac, i.e. mucosal or serosal surface, and suspended in normal saline. The bladders were placed in thyroxine at 10^{-6}M and the water losses were 39.08 ± 1.10 μl./cm^2./hr. for the everted bladder and 42.07 ± 1.57 μl./cm^2./hr. for the normal. There were no significant differences between the normal and everted bladders. Similar

experiments performed with triiodothyronine gave comparable results, that is, everted bladders 35.72 ± 2.6. µl./cm^2./hr. and 38.70 ± 1.86 µl./cm^2./hr. for the normal bladder.

3. Inhibition

Table 3 shows the effects of the presence of both thyroxine plus an inhibitor compared with thyroxine alone on water loss values across each separate lobe of the bilobed bladder from the same toad. All the inhibitors used reduced water loss from the bladders when in conjunction with thyroxine. When inhibitor alone was added to the bladder there was little or no effect on the water loss. Lowering the ambient temperature and perfusion with nitrogen reduced the effects of both thyroxine and triiodothyronine on water permeability. The triiodothyronine-induced water loss of 37.53 ± 2.71 (10) at 25°C. was reduced to 17.02 ± 1.5 (10) µl./cm^2./hr. at 4.5°C. and after nitrogen perfusion the triiodothyronine-induced water loss of 36.88 ± 1.02 (8), obtained under normal conditions, was reduced to 14.74 ± 1.02 (8) µl./cm^2./hr. During the experiments in which a half bladder was perfused with nitrogen, a glucose-free medium was used and this had no effect on the thyroxine or triiodothyronine induced increase in water loss under normal conditions. Hence the absence of glucose is not likely to affect the reduction in water loss caused by nitrogen perfusion alone. The results of using a 10^{-6}M potassium cyanide solution were rather variable, some bladders showing normal thyroxine-induced water losses and others being inhibited, such that the water loss in the latter case was reduced to control

Table 3. Effect of certain inhibitors on water loss from the isolated toad bladder.

	Water loss			
	Control	Inhibitor alone	Thyroxine + inhibitor	Thyroxine alone
Sodium arsenate (10)	12.82±1.12	8.63±0.89	18.00±0.93	38.72±1.99
Sodium azide (6)	11.48±0.57	10.83±1.26	11.88±1.76	39.36±2.50
Sodium malonate (10)	8.00±0.59	9.72±1.11	13.61±1.60	40.71±1.90
Sodium monoiodoacetate (8)	10.07±1.12	9.73±0.92	12.94±1.86	34.73±3.11
2,4-dinitrophenol (10)	11.61±0.96	9.57±0.86	18.72±0.67	40.02±1.77
Reduction of temp. to 4°5 C (12)	10.84±1.02	-	14.35±0.42	43.79±3.56
Presence of nitrogen	11.32±1.17	-	18.55±0.61	37.67±2.71

Values are given as means±s.e. in $\mu l./cm^2$. during first hour of incubation. Number of experiments in parentheses. In each case $P < 0.001$ for effect of inhibitor+thyroxine compared with thyroxine alone.

levels. A higher concentration, 10^{-5}M, was therefore employed and was effective in lowering the thyroxine induced increase to control levels, the values being, normal bladder plus thyroxine 38.74 ± 1.34 µl./cm^2./hr. and a similar bladder plus 10^{-5}M potassiu cyanide 14.01 ± 1.03 µl./cm^2./hr.

4. Sodium-free incubation medium.

Immersion in choline medium reduced the water transport of thyroxine-incubated bladders. Fig. 5 shows that although water loss in thyroxine-treated bladders is considerably inhibited there is still an increase which must be the effect of thyroxine. Despite this marked reduction in response the linearity of the dose-response relationship remains. The control values for both normal and choline media were of the same order, that is 11.54 ± 0.99 (8) µl./cm^2./hr. in normal saline and 10.54 ± 0.76 (8) µl./cm^2./hr. in choline saline. Although the thyroxine response is decreased, the time curve follows the pattern shown in Fig. 3.

5. Isosmotic conditions.

The effect 10^{-6}M solution of thyroxine on the toad bladder, divided for treatment as in Table 2 is shown in Table 4.

The addition of thyroxine to the serosal surface of the bladder causes a net movement of water to the serosal side. Thyroxine added to the mucosal side alone causes only a slight increase in water movement, but when on both sides of the bladder thyroxine results in a shift of water of about half that when thyroxine is on the serosal side alone.

With the bladder in the normal condition, when thyroxine is

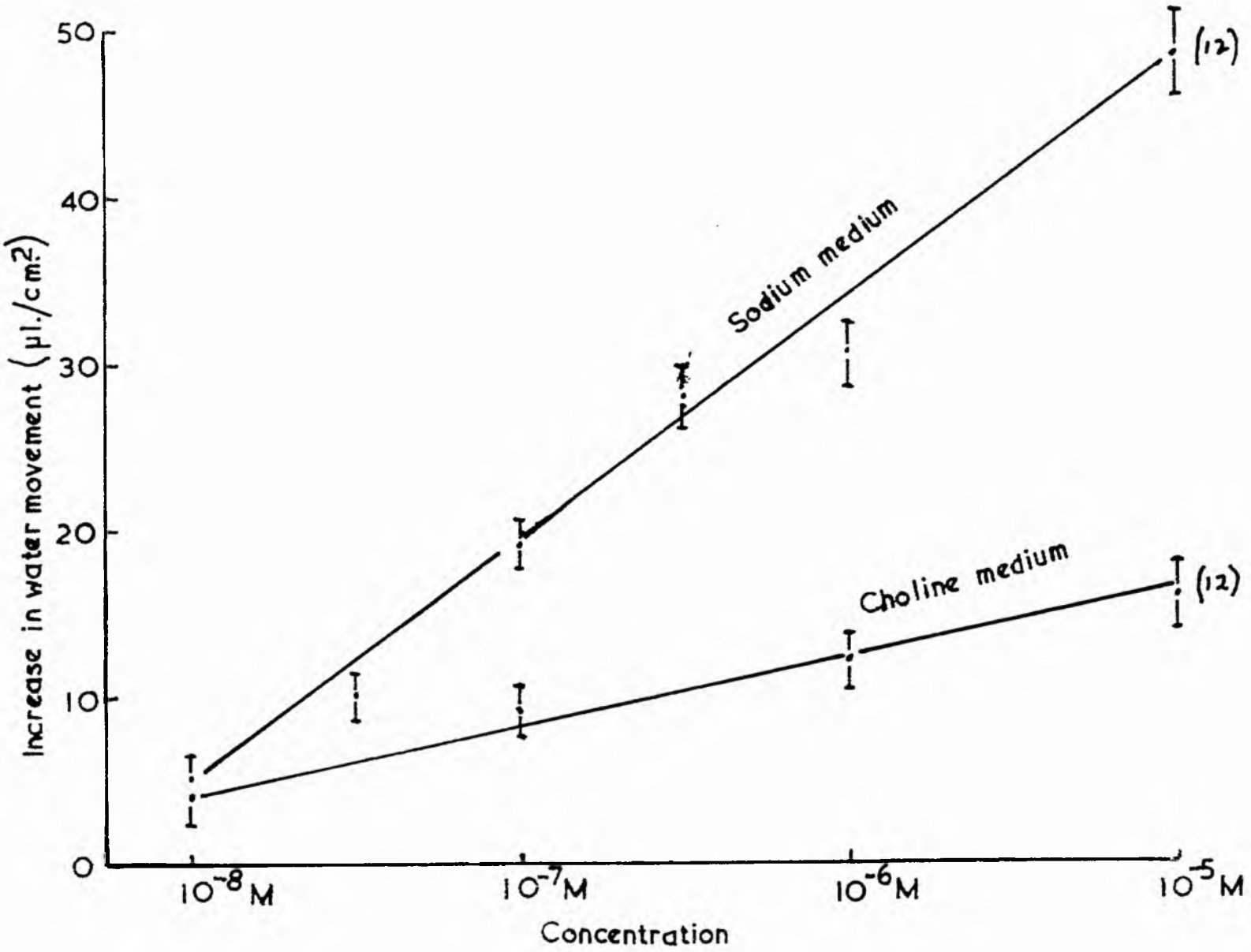


Fig. 5. Effects of different concentrations of thyroxine on water movement from the isolated toad bladder in both sodium and choline media.

Table 4. Effect of thyroxine (10^{-6}M) on water permeability of the isolated toad bladder under iso-osmotic conditions.

	Water movement ul. / $cm.^2$ / Hr.	Means of increases
Normal Condition		
Saline	2.49±0.44	11.02
Thyroxine; serosal	14.85±2.33	
Saline	2.44±0.54	6.75
Thyroxine; mucosal and serosal	8.55±2.62	
Saline	2.01±0.71	1.93
Thyroxine; mucosal	4.22±0.52	
Everted Condition		
Saline	1.63±0.71	10.87
Thyroxine; serosal	13.91±2.71	
Saline	1.93±0.63	6.21
Thyroxine; mucosal and serosal	8.38±1.99	
Saline	2.00±0.51	2.06
Thyroxine; mucosal	5.22±0.91	

24 half bladders (12 animals) in normal condition and 24 half bladders (12 animals) in everted condition divided as in Table 1. Values given as means±S.E.

applied to the mucosal side, there is only 200 - 400 µl. of saline and a correspondingly small amount of hormone inside the bladder sac. With the bladder in the everted condition, there is 15 ml. of saline surrounding the mucosal surface and although the hormone concentration is the same there is a greater absolute amount of thyroxine present. The results indicate that the effect is dependent upon the hormone concentration to which the bladder is exposed, rather than on the total amount. A similar result is obtained when the hormone is applied to the serosal surface. For although the water shift is the same in each case, with the bladder in the normal condition there is 15 ml. of hormone solution on this surface, whilst in the everted condition only 200 - 400 µl. are present on the serosal surface.

6. Re-incubation in triiodothyronine.

Fig. 6 shows the result of incubating three separate half bladders, each in the normal condition, at intervals of one hour in the same 10^{-6}M triiodothyronine solution. The first bladder (I) gave the typical response (see Section II,C,7), the second (II) one a slight response but the third (III) gave none at all. The procedure was repeated six times.

7. Thyroxine: Triiodothyronine response.

Fig. 7 shows the response to a thyroxine;triiodothyronine mixture. The water loss rises within the first twenty minutes and then falls slightly before increasing again. The initial rapid rise is very similar to that obtained in triiodothyronine alone and if the two separate graphs of water loss caused by

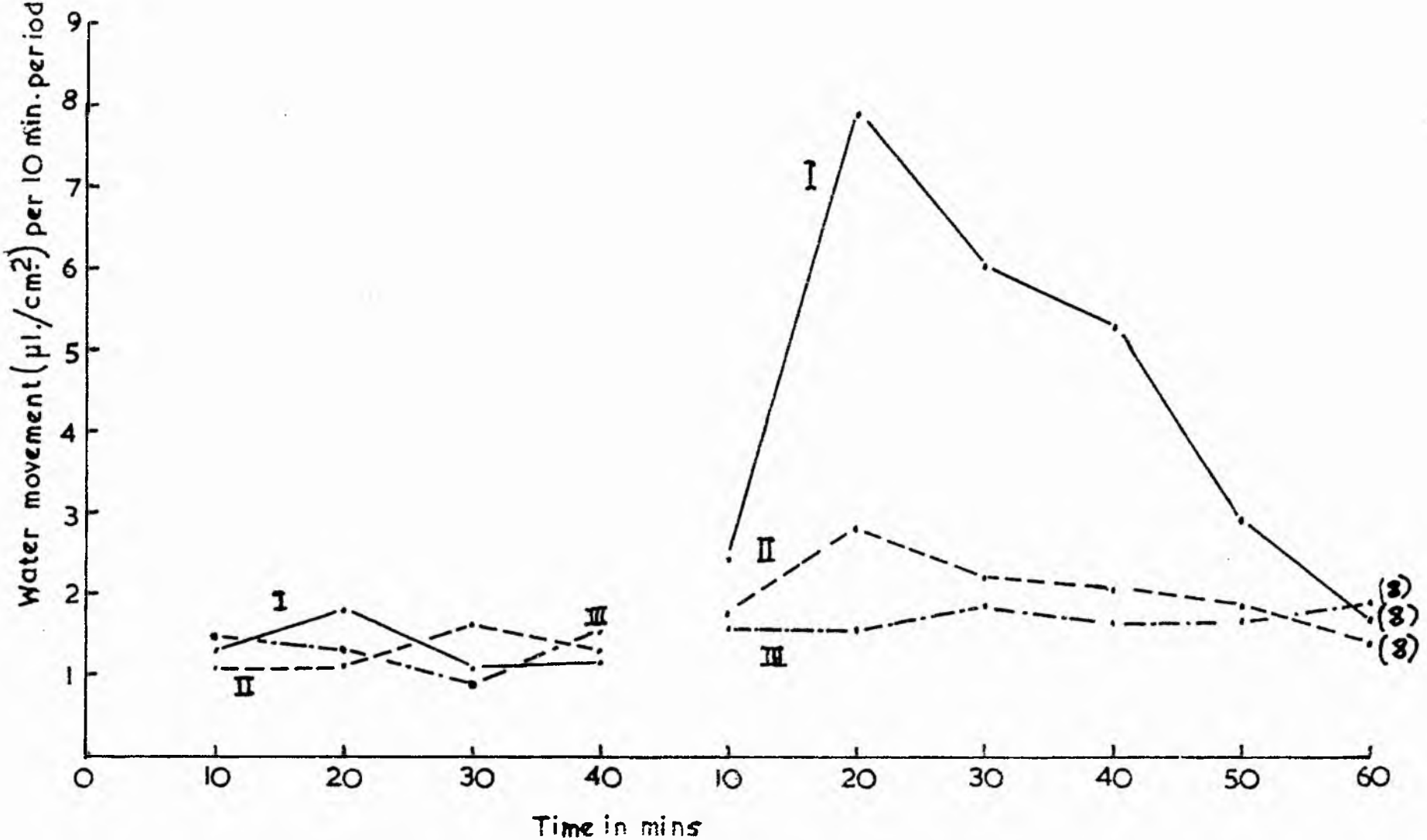


Fig. 6. Result of incubating successive half bladders (I,II,and III) in the same triiodothyronine medium (10^{-6} M).

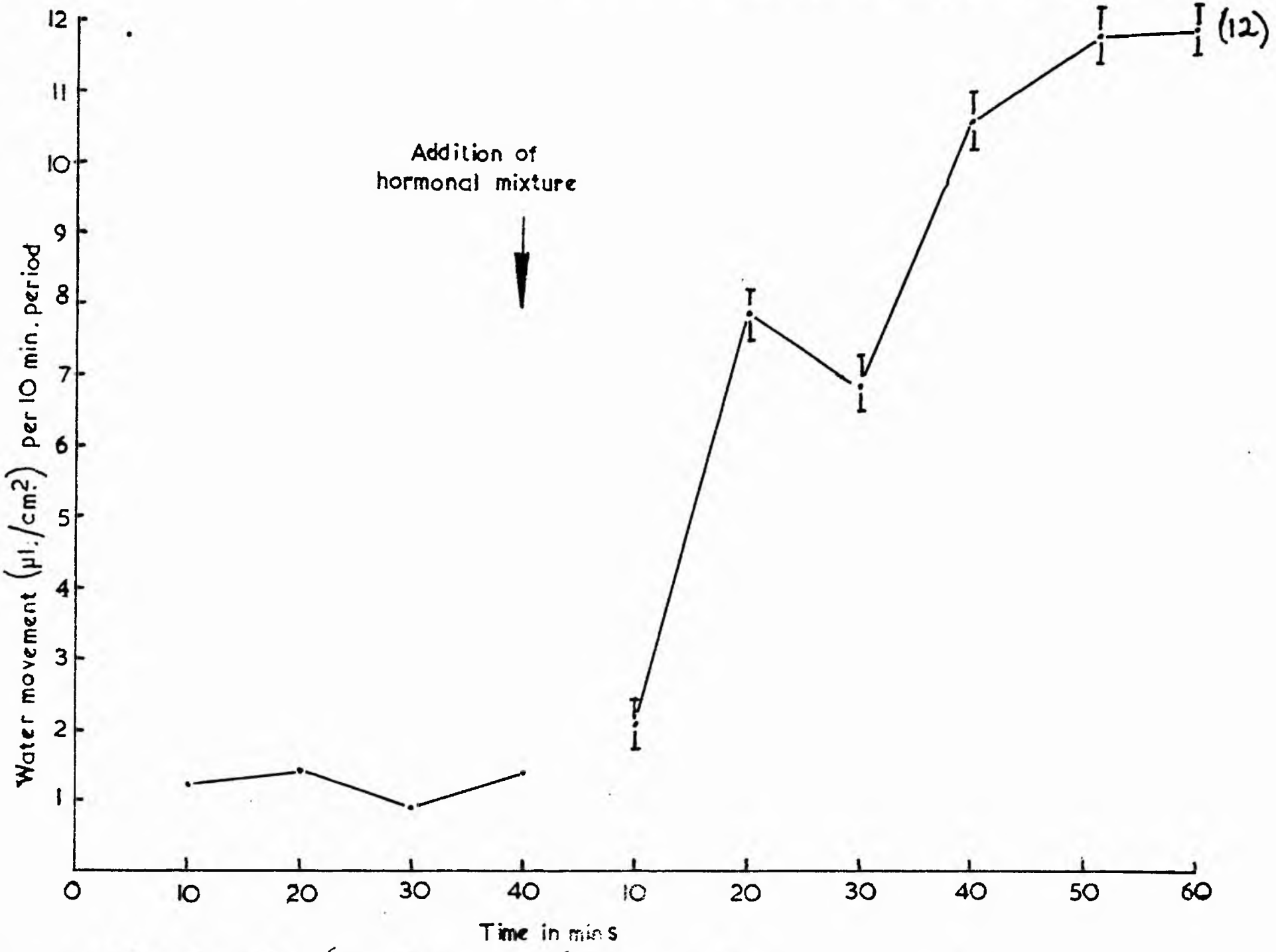


Fig. 7. Effect of 10^{-6} M thyroxine : 10^{-6} M triiodothyronine mixture on water movement across the isolated bladder of the toad.

thyroxine and triiodothyronine obtained in previous experiments are superimposed then a graph similar to that seen when both hormones are mixed is obtained (cf. Fig. 3). There appears, however to be a shift of the thyroxine curve nearer to the point of addition of the hormones.

8. Analogues

TRIAC and TETRAC, when applied to the serosal surface of the bladder, resulted in a very rapid increase in water loss (Fig. 8) It appears that TRIAC at 10^{-6}M has an immediate effect on the membrane causing increased water loss within two minutes. TETRAC at 10^{-6}M has a slower effect which is still very rapid compared with that of triiodothyronine and thyroxine. With both analogues water movement is reduced to control levels within 15-20 minutes. Neither tetraiodothyroformic acid nor tetraiodothyropropionic acid caused any increase in water movement down an osmotic gradient when administered to the serosal surface of the bladder. That the effect is real and not a consequence of changing the salines bathing each side of the bladder is borne out by experiments which were performed to test this possibility. The salines, both inside and outside the bladder were changed, and no hormonal addition was made and although weighings were continued at two minute intervals no change in water loss could be detected. This data, together with that obtained using the other analogues which had no effect on water movement, demonstrates that the effect obtained with TRIAC and TETRAC is a real one.

From these data a comparison was made of the total increase

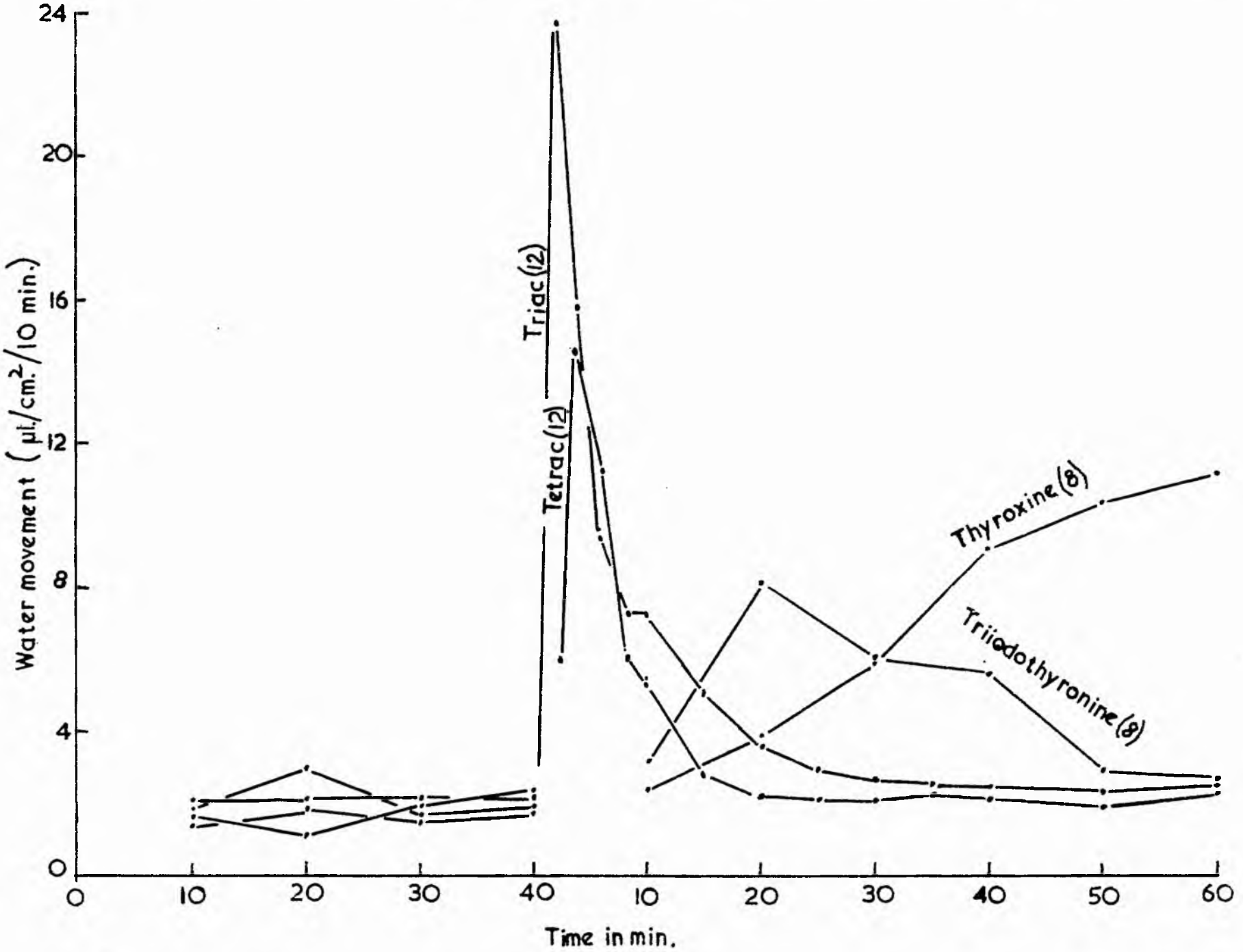


Fig. 8. Effect of TRIAC and TETRAC, plotted with triiodothyronine and thyroxine for comparison, on water movement (ul./cm.^{2} bladder/10 min.) across the isolated bladder of the toad.

in water loss brought about by TRIAC, TETRAC, triiodothyronine and thyroxine.

The results showed that thyroxine caused the greatest total increase in water loss, followed by triiodothyronine, TRIAC and TETRAC. In speed of action, TRIAC was fastest followed by TETRAC triiodothyronine, thyroxine and as regards maximal water loss per unit time, TRIAC caused the greatest loss followed by TETRAC, triiodothyronine and thyroxine.

D. DISCUSSION

Triiodothyronine and thyroxine when added to a physiological saline solution bathing the serosal surface of an isolated toad bladder increase the rate of water transfer across the wall from a hypotonic saline solution inside the bladder. The increase (five fold at the maximal concentration of hormone used), although considerable, is much less than that brought about by vasopressin acting on a similar membrane preparation (Bentley, 1958). The normal water movement of the bladder of Bufo bufo, which is dependent upon the osmotic gradient, is in these experiments slightly higher than that found by Bentley (1958) in B. marinus bladder under similar osmotic conditions, that is 11.5 μl./cm^2./hr. compared with 6.6 μl./cm^2./hr. This may not be of any physiological significance but may be due in large measure to the slight osmotic

differences between the saline used by Bentley and that described here.

The doses used in the experiment were of the same order as the concentration of thyroid hormones reported in the blood plasm of mammals (Pitt-Rivers and Tata, 1959) and, although it can be argued that mammalian requirements are higher due to their higher metabolic rate, it may be assumed with reasonable confidence that the doses are of a physiological level. Thyroid hormone concentrations in the blood of Amphibia appear not to have been investigated, except in one case - that of the male newt (Genest and Adams, 1957) and the concentration found was of the same order as that used in vitro in the present experiments. During the initi experiments a complete range of doses was used, but in later work when investigating an effect of the hormones a dose of 10^{-6}M was chosen, for this seemed to give suitable high responses to study and in addition did not represent a pharmacological dose. The different parameters of tissue activity which were measured showe only quantitative differences between 10^{-6}M and the lowest concentration studied, 10^{-8}M. Much of the work in the past in diff ent groups of animals may have given a false impression of thyroxine action because of the high doses employed. It is well know that thyroxine, in low concentrations has a stimulating effect, whilst at higher concentrations it has an inhibitory effect, and this has been shown on a number of parameters such as protein, lipid and glycogen metabolism (see Hoch, 1962). This also reconciles a number of reports on inconsistent and opposite actions

of the thyroid hormones.

Investigations on vasopressin and related substances have utilized the toad bladder largely because of the connection between the urinary bladder and the anti-diuretic action of the hormone (Bentley, 1958; Leaf, 1960a; Rasmussen, Schwartz, Schoessl and Hochster, 1960). Also, the analogy of the bladder to the kidney tubule which represents histologically and physiologically a far more complex structure has been drawn by Leaf (1960b). Work performed on the bladder has shown that the results obtained in response to the addition of various antidiuretic hormones resemble those seen after similar application to kidney tubules. The present experiments, however, were designed to use the bladder as a membrane with which to explore *in vitro* the effects of thyroid hormones on membranes. It must be emphasised that there is no justification for assuming that thyroxine exerts an anti-diuretic effect in adult toads and even if such an effect existed it would probably be obscured by the action of vasopressin.

It is apparent that triiodothyronine does not cause a greater maximum effect on water loss either in terms of total loss or as maximal loss per unit time. This is in contrast to studies on other parameters in mammalian tissue, where triiodothyronine produces not only a more rapid but also a greater maximal response than thyroxine *in vitro* (Gross and Pitt-Rivers, 1953; Barker, 1956). Work on uptake of thyroxine and triiodothyronine in dog liver (Roche and Michel, 1960) has shown that the triiodothyronine

penetration into cells is at least five times faster than thyroxin From this work it has been assumed that the ratio between the time necessary for the entry of triiodothyronine and of thyroxine into cells can partly explain the biological differences between the hormones. These authors have also shown that in the whole animal the situation is further complicated by the fact that triiodothyronine is bound to the plasma protein much more freely than is thyroxine, but that the phenomenon of entry into the cell is, in part, independent of this finding. This concept of rapid entry and metabolism was also proposed by Etling and Barker (1959) and Barker (1962) and the rapid water loss response of triiodothyronin appears to support this. The results of incubating successive bladders in the same triiodothyronine solution also leads to the same conclusion. The response of bladder 1 (see Fig. 6) is complete within an hour and the response of bladder 11 indicates that there is very little hormone remaining in the solution. It thus appears that all the hormone quickly enters the cell and is metabolised, or is rapidly adsorbed, as a protein-hormone complex, ont the cell surfaces either on receptor or non-receptor sites, before entering the cell.

The action of triiodothyronine has been shown to be rapid, whereas thyroxine has a much slower action on water movement across the isolated bladder. Fig. 7 indicates that although the two hormones differ in speed of action they can act together. There is a significant shift of the thyroxine part of the combined curve in Fig. 7 towards the time of addition of the two hormones.

This suggests that either the entry of thyroxine into the cell is facilitated by triiodothyronine, or that attachment to sites on the cell surface is increased. The alteration in the thyroxine part of the curve provides more evidence that these hormones are affecting permeability of the membrane and of the cells which constitute the membrane.

Several chemicals known to inhibit specific enzyme systems involved in metabolism reduced the water loss from the isolated toad bladder in the presence of thyroxine. It would seem from the results using inhibitors, which exert their effect on both oxidative and glycolytic systems, that the increase in water movement brought about by thyroxine is dependent upon various mechanisms of energy transfer within the cells of the bladder. For instance, sodium arsenate uncouples phosphorylation processes within the mitochondria and inhibits the exchange of adenosine triphosphate and inorganic phosphate, thus eliminating reformation of adenosine diphosphate; sodium azide exerts a similar effect and in addition inhibits cytochrome oxidase activity. Dinitrophenol, uncouples phosphorylation and inhibits the exchange reactions of adenosine triphosphate, as well as stimulating adenosine triphosphatase activity; sodium malonate inhibits succinic dehydrogenase whilst sodium monoiodoacetate acts as an inhibitor of the glycolytic phase of cellular metabolism (Baldwin 1959; Lehninger, Wadkins and Remmert, 1958; Drebs, 1954). Thus, sodium azide and malonate inhibit aerobic metabolism, while monoiodoacetic

acid and dinitrophenol inhibit glycolytic systems within the cell. Inhibition of general metabolism lowers the thyroxine-induced water loss and thus it appears that metabolic energy is stimulated by thyroxine to bring about the water loss response. It would appear that the transfer of water brought about by thyroxine is, in some way, dependent upon both glycolytic and oxidative energy supplies for the fulfillment of the hormonal action. These results are in contrast to those of Rasmussen, Schwartz, Schoessler and Hochster, using the urinary bladder of B. marinus, who showed that arginine vasopressin increased the water permeability of the bladder kept at 2.5°C., under nitrogen perfusion and in choline medium. They concluded that the permeability of the cells was not directly linked with metabolism. Although the present studie indicate that thyroxine may exert a control of water permeability in the stimulated state it is not known how this is achieved, one can only surmise that the increased metabolic state of the cells causes an opening of pores within the membrane which enables a more free transfer of water. Active sodium transport in the isolated toad bladder appears to be dependent on oxidative metabolism (Leaf, 1960a) and the presence of sodium has been shown to potentiate water movement in response to thyroxine. Replacement of sodium in the medium by choline reduces the increase in water permeability normally induced by a range of doses of thyroxine. Thus the increase in water shift may be a reflection of a more general increase in permeability brought about by thyroxine.

Permeability to water is increased across the toad bladder

by vasopressin but there is little effect on general cellular metabolism, although active transport of sodium, which is an energy-dependent process, is increased. Also bladder-membrane pore size, which will itself increase availability of substrates, including sodium ions, for metabolism, appears to be regulated by vasopressin (Leaf, 1961). The increase in respiration rate brought about by vasopressin in sodium medium is of the order of 30%, whilst there is no increase in the first hour after addition of this hormone to bladders incubated in a sodium-free medium (Leaf, 1961). The results of further studies with thyroxine (see IV,C) indicate that oxygen uptake is stimulated by approximately 100%, both in the presence of and absence of sodium. Thu thyroxine, unlike vasopressin, does not appear to be restricted i its control of metabolic processes. This effect is probably due to the fact that thyroxine neither directly affects pore size nor is it specifically directed to sodium kinetics, but has a generalized role in stimulating enzyme systems.

In order to obtain reasonably large increases in water transfer most of the work reported was performed using an osmotic gradient; some experiments, however, were made in the absence of an osmotic gradient. These experiments demonstrate that, at least within the first hour of application of the hormone, the absolute amount presented to the bladder is unimportant as regards response The response being dependent upon concentration of the hormone.

The results show that thyroxine accelerates the movement of

water even in the absence of an osmotic gradient, as seen in both the normal and everted condition. When thyroxine is applied to the serosal surface, however, the water movement values are higher than any others and this may be compared with work on the influence of thyroxine on the potential and short-circuit current of the toad bladder (III,C). Thyroxine applied to the serosal surface of the bladder causes an increase in short-circuit current, a direct measure of active sodium transport in this membrane, but the potential remains virtually constant. An increased movement of anions which maintains a steady potential has been shown to occur (III,C) and this implies an effect of thyroxine on the permeability of the membrane apart from its stimulatory effect on active sodium transport. The present experiments are supported by this concept, since with increased movement of ions from mucosal to serosal fluids there would be a corresponding shift of water due to the increased osmotic activity of the serosal fluid. In a similar manner the effect of frictional drag of solvent molecules on solute ions would tend to move more anions (Koefoed-Johnsen and Ussing , 1953). Thyroxine, therefore, appears to increase the area available for diffusion through the membrane and allow freer exchange of substances between the media bathing each surface of the membrane. Vasopressin has been shown to produce a similar effect on the bladder of Bufo marinus (Hays and Leaf, 1962b) where further analysis has indicated that the hormone enlarges the aqueous channels in the membrane. The core of water they contain possesses the physical properties of ordinary bulk

water under these conditions, unlike that of the control condition where the water in the pores is in a structured state.

The behaviour of the everted toad bladder, when an osmotic gradient exists from the serosal (hypotonic) to mucosal (isotonic) surfaces is markedly different from that of vasopressin treatment. Whereas vasopressin elicits an effect on water transport only when presented to the serosal surface of the bladder (Leaf, 1960a), thyroxine under these experimental conditions acts on both surfaces with equal facility. In the presence of an osmotic gradient it is apparent that a limit of pore size has been reached when 10^{-6}M concentration of thyroxine is administered to either side of the membrane. Thus allowing the water loss values, obtained with an osmotic gradient of 206 milliosmoles, to be dependent upon the relationship between the pore size which is achieved and the osmotic gradient alone. The data suggests, however, that at 10^{-6}M concentration, thyroxine opens the hypothetical 'pores' when under isosmotic conditions to the same extent as it does when applied to a bladder across which a transmembrane osmotic gradient exists. The effect of the ionic movement in the former case, however, provides the bias from the mucosal to the serosal surfaces. In addition, if the permeability barrier to water is located at the mucosal surface, as has been suggested by the work of Leaf (1960a), then it is readily seen that thyroxine applied to the serosal surface could firstly stimulate the active transport mechanism, which is located at the serosal surface, before penetrating the cell and affecting the mucosal barrier.

When placed on the mucosal surface alone, thyroxine causes but a slight increase in water movement, which may probably be explained by a form of mucosal barrier similar to that proposed by Leaf (1960a). The effect of this barrier being to give thyroxine a lower penetration rate through this surface of the membrane than through the serosal surface. Thyroxine applied to the mucosal surface would first have to penetrate the permeability barrier which exists to solutes, before penetrating the cell further and finally acting on the serosal pump mechanism. The water transfer figures therefore offer an indication of the rate of penetration of thyroxine through the respective surfaces of the cell. Such values, although useful, must be treated with respect for, as the data shows, the thyroxine effect is completed within one hundred and thirty minutes and one must keep in mind the fact that during these experiments thyroxine is being utilised by the cell, thereby depleting the amounts still available.

When analogues of the thyroid hormones were first discovered they were thought to be the active form of the hormones, but despite the vast amount of work on these forms, more recent reviews (Pitt-Rivers and Tata, 1959; Tata, 1961) have tended to discount this view. The increase in water movement by the toad bladder occurs more rapidly with the analogues TETRAC and TRIAC than with the parent hormones, however, and is suggestive of biological activity. Neither tetraiodothyroformic acid nor tetraiodothyropoprionic acid, despite their proven activity in other assay techniques (Bruice, Winzler and Kharasch, 1954; also Pitt-Rivers

and Tata, 1959, pp.93-96), have any effect on water permeability across the isolated toad bladder. The triiodo-compounds (triiodothyronine and TRIAC) act more quickly than do the corresponding tetraiodo-compounds (thyroxine and TETRAC). The maximum rate of water loss is greatest for TRIAC, decreasing through TETRAC and triiodothyronine to thyroxine, while thyroxine causes the greatest total loss followed by triiodothyronine, TRIAC and TETRAC. It appears, therfore, that the triiodo compounds enter the cell more quickly and are metabolised faster than the tetraiodo compounds, a finding supported by previous mammalian work in vitro (Barker, 1956). This work on the toad bladder enables one to show that there exist differences in the biological activities of the parent hormones and the analogues, although giving no indication as to their cellular site of action. The results show slight differences from results on similar assays in other groups. The acetic acid analogues for instance, as can be seen in Pitt-Rivers and Tata, (1959), were shown in the tadpole growth assay to be ten times (TETRAC) and twenty times (TRIAC) as active as L-thyroxine. Whereas in the present experiments, although showing a much faster action, they both cause low total water loss compared to the parent hormones.

These experiments show for the first time that the thyroid hormones influence permeability, thus bringing them into a similar category as other hormones such as insulin, vasopressin and oestrogens which are all thought to bring about their effects by altering permeability (Hechter and Lester, 1960). Whereas

insulin and other hormones seem to affect the permeability of the cell membranes directly, it might appear from the present results that the action of thyroid hormones on cells is to bring about its manifold effect on permeability processes by means of its effects on enzymic processes which further control more general permeability. The recent work of Tata (1962) has shown, on the contrary, that in mammalian tissue, alterations in penetration of labelled amino acids into cells precedes an increase in metabolism, and any effects on enzyme systems. Thus, if the extrapolation into the amphibia is valid, it would appear that the increase in enzyme activity is possibly secondary to the basic action on the cell membranes, although inhibition of the enzymes in the present experiments reduces the thyroxine-induces water loss. This can readily be explained by the fact that under normal conditions enzymes are limited by the amount of substrate available and do not normally work at their maxima due to this. If the rate of entry of substrates into the cell is increased this would itself stimulate enzyme processes, without requiring any direct hormone-enzyme interaction. Thus permeability effects of the hormone may, as a result of its effect on availability of substrates, appear to be linked with metabolic effects. The fact that the thyroxine induced water loss is reduced when inhibitors of enzyme processes are used need not, therefore, necessarily mean that the thyroid hormones are affecting enzymes directly. Rather that the hormonal effect on cellular permeabilit is masked by the restriction placed upon cellular metabolism by

the inhibitors, which would further control the more general permeability of the total membrane. The effect of thyroid hormone on cellular permeability appears therefore to be related to an increased metabolic state within the cell, which further causes an effect on the permeability of the tissue as a shole, such as alteration of 'pore' size. The possibility remains that the thyroid hormones may alter cellular permeability and hence bring about the great diversity of metabolic actions that have been seen as a consequence of their presence in the living vertebrate.

SECTION III

Effect of thyroidal hormones on ionic permeability of toad bladder and skin.

A. INTRODUCTION

It was originally thought that the membranes of cells were impermeable to solutes since the ionic content of cells remains virtually constant despite changes in the surrounding fluid. With the advent of isotopic techniques, however, it was readily shown that such membranes were highly permeable to many substances, including water and electrolytes as well as organic molecules, ... Such studies were continued and suitably demonstrated that the ionic consistency of cells was maintained by a movement of ions into and out of the cell. Much useful information as regards ion transport has been obtained from studies in nerves (Huxley, 1959), frog skin (Ussing and Zerahn, 1951), gastric mucosa (Durbin, Curran and Solomon, 1958; Forte, Adams and Davies, 1963), rat ileum (Curran and Solomon, 1957), rat colon (Curran and Schwartz, 1960), toad large intestine and guinea-pig caecum (Ussing and Anderson, 1955), teleost intestine (House and Green, 1963) and kidney tubules (Giebisch, 1962; Ullrich and Marsh, 1963) to mention only a few of the membranes studied. Such studies are, however, somewhat complicated by the many layers which constitute these membranes making them unsuitable for studies on basic ion and molecular transport processes and more so when hormonal action is being investigated. More structurally simple membranes have therefore been sought, which also transport ions in a similar manner to those membranes described above.

The mechanisms by which inorganic ions and organic substances across cell membranes have been the subject of much intense work and two of the objects which have been a favourite among students of ion transport have been the iolated surviving toad and frog urinary bladder and skin. These membranes are particularly suited for such studies since they survive for long periods in artificial media resembling the Amphibian plasma. In addition, they are both interesting in their own rights and also because there is mounting evidence that they can serve as model systems for the study of properties found in many epithelial and glandular tissues.

The isolated toad urinary bladder fulfills the requirements of structural simplicity (see I) and despite its transparent thinness a bioelectric potential of some ten to fifty millivolts can be measured; with the serosal or blood side being electropositive with respect to the mucosal surface. The toad skin, although possessing secretory glands on the mucosal surface, also has the advantage, in a manner similar to the bladder, that it is of simple structure with only a very few intracellular compartments. The study of ionic transport particularly that of sodium, across these, as well as other membranes, has progressed rapidly through the work of Ussing and his associates especially since their introduction of the short-circuit current technique.

Besides maintaining a potential difference, even when in contact with media containing identical amounts of sodium on each surface, these amphibian membranes have another striking property, namely that of transporting sodium chloride from the outside to the inside solution (Huf, 1935, 1936; Krogh, 1937; Leaf, 1955; Leaf,

Anderson and Page, 1958). As well as these *in vitro* measurements, potentials have been measured *in vivo* (Kalman and Ussing, 1954; Brown, 1962), which confirm the validity of earlier measurements of salt uptake on whole animals (Krogh, 1939) and also indicate the reality of *in vitro* potential measurements. That sodium transfer is due to active transport is evidenced from the fact that it can take place against an electric as well as a chemical potential gradient (Ussing, 1949 a; Leaf, Anderson and Page, 1958). The chloride transfer, however, always takes place down the electrochemical potential gradient. Indeed the ratio between the inward and outward fluxes of chloride ions is always exactly what would be predicted from the single flux equation (Ussing, 1949 b) for an ion moving passively through the membrane, influenced only by the combined effect of concentration and electrical gradients (Koefoed-Johnsen, Levi and Ussing, 1952; Leaf and Hays, 1962). Thus from the following equation:-

$$\frac{M_{in}}{M_{out}} = \frac{C_o}{C_i} \exp. \frac{zFE}{RT}$$

Where M_{in} is the inward flux, M_{out} the outward flux, C_o is the concentration of the ion in the outside solution, C_i its concentration in the inside solution, E is the potential difference between the inside and outside solutions, and z, R, T and F have their usual meanings (see Ussing and Zerahn, 1951). It can readily be found from isotopic and potential measurements whether an ion is actively transported. If the ratio is greater than one then it may be that the ion is transported if there is no hydrostatic

pressure difference between the two solutions bathing the membrane Also, given that the activity coefficients of the two phases on either side of the membrane are the same, a given ion will be in equilibrium with respect to a given membrane when its electrochemical potential in the outside solution $\bar{\mu}$ (o) equals its electrochemical potential in the inside solution $\bar{\mu}$ (i). The electrochemical potential of the jth ion is given by:-

$$u_j = RTln a_j + zF\psi + I.$$

where R, T, z and F have their conventional meaning, a denotes chemical activity, ψ electrical potential and I a constant. Deviations from the equilibrium condition

$$\bar{\mu}\ j\ (o) = \bar{\mu}\ j\ (i)$$

are indications that the ion is subject to active transport.

The fact that sodium transport is active whereas the chloride movement is passive suggests that the potential driving the chloride ions through the frog skin is created by the active sodium transport (Ussing, 1949 b). Conclusive proof of this hypothesis was obtained by way of the short-circuiting technique used in conjunction with isotopic measurements in frog skin by Ussing and Zerahn (1951), and in toad bladder by Leaf, Anderson and Page (1958). When a potential across a piece of bladder or skin, with identical sodium-containing solutions on both sides, is maintained at zero by an applied E.M.F., the current generated by the skin or bladder is exactly equal to the net flux of sodium ions transported from the outside to the inside. Chloride ions, under the same conditions, pass through the membrane in both directions at

exactly the same rate, thus giving no contribution to the flow of electric current. Linderholm (1952) fully confirmed these findings in frog skin, while the present findings are in agreement with those on toad bladder.

The transfer of sodium ions is quite clearly active, since the inward flux is from ten to fifty times greater than the outward flux (as determined by measurement of the two fluxes with isotopic tracers of sodium). Thermodynamically the work involved when sodium ions are transported between solutions of equal composition and equal potential is nil, but during transport the sodium ions must overcome the frictional resistance of the cell membranes and other structures in the membrane. The overcoming of the resistances represents the work done in the short-circuited skin. Thus the total electric asymmetry of the isolated surviving membranes (when in contact with identical solutions) comes from the active transport of sodium ions from outside to inside. The chloride (and other passively diffusing ions) presents a shunt, tending to lower the voltage of the total system below the ideal E.M.F. of the sodium 'battery'.

Transport of a substance across a biological membrane is called passive if the process can be accounted for by means of ordinary physical forces: those caused by a concentration gradient, an electrical gradient, a solvent drag or any combination of these. Active transport processes, then, are those which cannot be explained as a result of these forces, but involving the participation of some energy-yielding chemical reaction. Active transport

processes have already been classified (Ussing, 1949 c; Rosenberg, 1954; Scheer, 1958) as those where the substance in questio is moved against an electrochemical gradient, that is, the substance gains free energy by the transfering process, which is derived from cellular metabolism. The demonstration of uphill transport, against a chemical gradient in the case of an uncharged particle and an electrochemical potential in the case of a charged substance, has generally been used as a criterion of active transport, since this is based on experimental evidence and is not concerned with differences in membrane structure.

That active transport processes are involved in a number of cases where ion accumulation and extrusion take place against thei electrochemical potentials is beyond doubt (see Koefoed-Johnsen and Ussing, 1960). The energy required for these processes has also been unequivocally shown to be derived from either anaerobic or earobic metabolism (Ussing, 1960 c). There have been several theories proposed to account for ion transfer and its metabolic requirements, but so far none has been proven. One of the earlie methods proposed was that a carrier system existed in the membrane which picked up the ions at one border and moved under the influence of a concentration gradient within the cell to the other border where the ion-carrier complex was destroyed and the carrier broken down. Thus the existence of complete carrier at one borde and the breakdown at the other created an intracellular concentration gradient. This theory, however, is insufficient to explain the strong discrimination shown by most membranes between sodium and potassium.

Another theory is based on the idea that there are several

sizes of pores in the membrane which allow only one type of ion or molecule to pass through them. It remains, however, to show how the specificity which membranes show to various ions could be attained by such a method.

Conway (1953) has proposed a transport system based on a cation complex formed with the reduced form of a carrier at one border, the carrier-cation complex then crosses to another border where oxidation takes place and the cation is released. Although this theory could be related to the specificity of ion transport, it relies on the fact that not more than four equivalents of ion could be transported per molecule of oxygen consumed according to the reaction:-

$$4H + 4e + O_2 \longrightarrow 2H_2O$$

There is much evidence that the ratio of equivalents of ions to the number of oxygen molecules consumed is much greater than four in frog skin, (Zerahn, 1956; Leaf and Renshaw, 1957), in frog mucosa (Crane and Davies, 1951, Davenport and Chavre, 1952), and in kidney tubules, (Lassen and Thaysen, 1961). Ratios greater than twelve equivalents of sodium transported per molecule of oxygen used have been found in all these tissues and in kidney tubule the ratio is as high as 28:1.

There have been several other theories, including one involving a contractile protein (Goldacre, 1952) which, it was proposed, worked by the coiling and uncoiling of peptide chains enabling the protein alternately to absorb and release ions.

The detailed work of Maffly and Edelman (1963) has indicated that the coupling of sodium transport to metabolism is achieved by

a highly organised molecular arrangement, with the metabolic systems in close proximity to, or even being part of, the transport mechanism. Thus the transport system is envisaged as being, at least in part, independent of the general metabolic pool. High energy phosphate molecules, such as adenosine triphosphate, have also been implicated as a source of energy for active transport processes (see reviews by Heinz, 1963, and Wilbrandt, 1963). The demonstration of sodium and potassium activated enzymes, such as adenosine triphosphatase, in various tissues, such as crab nerve (Skou, 1957, 1960, 1961), crab gill (Schoffeniels, 1962), brain microsomes (Jarnefelt, 1962 a,b) and brain and kidney (Skou, 1962) has added further support to this concept. The work of Judah and Ahmed (1962), Judah, Ahmed and McLean (1962) and Ahmed, Judah and Tallgreen (1963) has pointed more to the influence of phosphoproteins in sodium transport mechanism. Certain drugs which, for instance, affect sodium transport prevent phosphoprotein turnover while having no immediate action on adenosine triphosphate. They have therefore suggested that the phosphorylation of phosphoprotein is the first step in this utilisation of adenosine triphosphate. Thus work on a variety of tissues has implicated these high energy compounds as an energy source for transport processes and the available evidence allows the speculation that part of the cellular energy derived from those high energy compounds is directed towards sodium transport.

Recent research on ion movements in many tissues has yielded evidence that sodium extrusion is not an independent process, but seems more or less firmly linked to potassium accumulation. This has been ably demonstrated in human red blood cells by Glynn (1956

frog muscles (Keynes, 1954) and frog skin (Koefoed-Johnsen, 1955; Koefoed-Johnsen and Ussing, 1956, 1958). It has already been shown that the potential across frog skin is the result of active sodium transport and the shunting by the anions (Ussing, 1948; Ussing and Zerahn, 1951), therefore, in order to study the sodium potential, it is necessary to remove the anion shunt. This may be effectively performed by substituting the non-penetrating sulphate ion for chloride. In the absence of such anions, the outside surface of frog skin behaves like a sodium electrode, whilst the inside acts like a potassium electrode. Thus, the outwards facing epithelium of the transporting cells appears to be selectively permeable to sodium ions whilst the inward-facing membrane is selectively permeable to potassium. The permeability of the inner cell membrane to free sodium ions (as distinct from those passing by the 'pump') is low. The active sodium transport mechanism is proposed to be located at the inward-facing membrane and to be a forced exchange of sodium from the cell with potassium from the inside bathing solution (Ussing, 1960 b). The pump is, therfore, electrically neutral. Sodium diffuses from the solution outside into the cell creating a diffusion potential E_o, the magnitude of which is given by the Nernst equation:-

$$E = \frac{RT}{zF} \log n \frac{Na(o)}{Na(cell)}$$

where R, T, z and F have their convential meanings, Na_o is sodium concentration in the outside solution and $Na(cell)$ is sodium concentration in the cell. Sodium is then exchanged for potassium from the inside bathing solution and potassium then diffuses from

the cell to the inside solution and creates a further diffusion potential, which can be expressed by:-

$$E_i = \frac{RT}{zF} \log_n \frac{K(cell)}{K(i)}$$

The total potential is therefore $E = E_o + E_i$

The development of microelectrodes (Ling and Gerard, 1949) has made possible measurements of transmembrane potentials of cells, and Engbaek and Hoshiko (1957) have shown the existence of the two predicted potentials in skin bathed in sulphate Ringer. It has, however, been shown that when the mucosal surface of frog skin is subjected to a potassium Ringer and the serosal surface is bathed by a sodium Ringer a net sodium flux persists and, indeed, exceeds the measured short-circuit current. The cumulative observations of the studies of Bricker, Biber and Ussing (1963) have led them to suggest that the possibility exists that the active transport of sodium may be an electrogenic process, although the demonstration of this process is experimentally difficult, and the movement of all ion species was not documented.

In toad bladder the evidence seems a little confused. Hays and Leaf (1961) showed that when potassium is removed from the serosal bathing fluid the active sodium transport is suppressed. Frazier (1962) using microelectrodes also showed the existence of the two predicted potential steps, and these findings are consistent with the Ussing hypothesis. In addition, Gatzy and Clarkson (1962) by measuring relative permeabilities of toad bladder surfaces found that their results on bladder were virtually the same as those of Koefoed-Johnsen and Ussing on frog skin. This evidence

supports the view that there is a forced exchange of sodium and potassium at the serosal surface. Essig, Frazier and Leaf (1963) and Frazier and Leaf (1963) have, however, from more recent studies, suggested that under certain conditions the transfer of sodium across the serosal surface is electrogenic, that is, the pump causes transfer of sodium from the cell to the serosal medium without the simultaneous and obligatory transfer of an anion in the same direction, or of a different cation in the opposite direction, as an integral part of the active transport step. The authors, however, seem a little unsure of the interpretation of their results which by no means demonstrate that the Ussing model does not apply to toad bladder. We must, therefore, await further experimental reports from Leaf and his co-workers on this interesting subject. There is, I feel at present, sufficient experimental evidence that the Ussing model applies to toad bladder as it does to frog skin.

Using the criterion of Ussing (1948) for active transport, Leaf (1960 a) and his co-workers have shown that many substances move passively through the amphibian urinary bladder and that sodium is actively transported from the mucosal to serosal surfaces. The normal physiological studies on ion movements across these membranes are sufficiently well documented to enable work on the effects of certain compounds on various parameters of activity to be performed with confidence.

Such studies have been persued by Bentley (1960), Sawyer (1961 a,b), Leaf and his associates (1958, 1960, 1961) on toad and frog bladder and by Ussing and Zerahn (1951) and Morel (1962) on skin.

These workers have particularly investigated the effects of posterior pituitary hormones on the ion movements across the membranes. Other hormones, for instance, adrenaline, whilst increasing chloride movement from serosal to mucosal surfaces by stimulation of the mucous glands (Koefoed-Johnsen, Ussing and Zerahn, 1952), has no effect on ion movement across toad bladder (Leaf, 1960 a). Leaf (see Goodfriend and Kirkpatrick, 1963) has shown that insulin increases the transport of sodium in the bladder, while Herrera, Whittembury and Planchart (1963) have similarly demonstrated that insulin increases active transport of sodium across frog skin. In addition, insulin treatment produces an increase in the potential difference of frog gastric mucosa, which is a reflection of the effect of the hormone on the chloride pump (Rehm, Schumann and Heinz, 1961).

In addition, steroid hormone effects on ion transport have been investigated by Schoeffeniels and Baillien (1960), who, after previous injection with male and female sex hormones, found increases of frog skin potential measured <u>in vitro</u>. McAfee and Locke (1961) have also demonstrated that certain steroids exert a control over the potential across frog skin. Whilst Taubenhaus, Fritz and Morton (1956) investigating the effects of steroids on frog skin, found that the hormones caused a gain of electrolytes into skin sacs made from frog hind legs, as measured by increasing depression of the cryoscopic constant of the inside bathing solution. It is therefore apparent that the presence of the s steroids used, when bathing the serosal surface of the skin, caused an increase in transfer of ions through the skin from the mucosal

to serosal surfaces. In addition, Bishop, Mumbach and Scheer (1961) have shown that prior destruction of the interrenal tissue results in a reduction in active sodium transport across the isolated frog skin, combined with an increase in the d.c. resistance; similar effects after adrenalectomy of frogs have been reported by Williams and Angerer (1959). Jørgensen (1950) reported an increase in short-circuit current of isolated frog skin, as a result of placing frogs in distilled water prior to removal of the tissue. This procedure brings about an increase in the secretion rate of aldosterone and the increase in sodium transport is ascribed to this. Penney, McAfee and Locke (1961) and Crabbé (1961 b) have shown that administration of aldosterone *in vitro* increases active sodium transport in frog skin and toad bladder respectively. Maetz, Jard and Morel (1958), from their *in vitro* work on frog skin, have reached conclusions similar to those of Penney and coworkers. Crabbé (1961 b), however, has reported that deoxycorticosterone has no *in vitro* effect on the sodium transport across toad bladder, (confirming the negative results obtained by Levinsky (quoted by Sawyer, 1956)), but prior injection of this hormone results in an increased short-circuit current, as measured *in vitro*.

Studies of hormone action on active transport mechanisms have not been confined to Amphibian tissues, however, for Aulsebrook (1961) has shown that vasopressin, when applied to the serosal surface of the rat colon *in vitro*, causes a stimulation in sodium transport. French and Manery (1962), working on adrenalectomised rats, found that subsequent injection of aldosterone restored the sodium/potassium balance of tissues. Low sodium content and re-

tention of potassium in rat diaphragm muscle cells are caused by addition of insulin to the bathing medium (Creese, d'Silva and Northover, 1958), whilst insulin addition to both rat skeletal muscle (Zierler, 1956) and rat adipose tissue (Beigelman and Hollander, 1962) causes an increase in the resting potential. Kerman (1961) found a closer agreement between the potentials calculated by the Nernst equation, and those found by micro-electrode techniques after addition of insulin to frog muscle, than that found in control muscle. Gimeno, Gimeno and Webb (1963), whilst studying the effects of testosterone and progesterone on rat atria, found that these steroids at 10^{-7}M produced a slowing of the depolarization of the action potential. These changes are a result of effects on the transmembrane fluxes of sodium and potassium, a decrease in permeability being assumed. In contrast to the work on frog skin and the bladder of the toad, aldosterone applied <u>in vitro</u> to the rabbit cornea has no effect on electrical activity (Donn, Rosenblatt and Christy, 1961). D-aldosterone, when applied directly to the tortoise bladder, causes an increase in short-circuit current, but produces no effect on the isolated intestine of this animal (Bentley, 1962) and this author also showed that the neurohypophyseal hormones have no effect on these membranes. When the perfused cat kidney is brought into contact with blood from intact animals containing aldosterone, the rate of distal sodium resorption and the proximal water-linked resorption of salt and water are increased (de Lima and Lockett, 1963). There also exist numerous reports from work on whole animals and from

clinical data that hormones affect the permeability of cells and tissues (see Lipsett, Schwartz and Thorn, 1961).

Data which exists on the direct effect of thyroid hormones on ionic movement is scarce. There have, however, been some reports of clinical observations on these hormones and their effects on ionic balance. Widjeveldt and Jansen (1960) have reported that there is an impairment of renal concentration processes in hyperthyroidism, resulting in increased losses of ions, and Boekelman (1958) found that hyperthyroid patients showed an increase in sodium content of erythrocytes. The report of Byrom (1933-34), as does that of Thompson (1925-26), indicates that resorption processes of the kidney are reduced in hyperthyroidism. Recently Satoyoshi et al (1963) has proved decreased potassium and water content and accumulation of sodium in muscles in thyrotoxic patients. One interesting piece of work by Hsu and Oliner (1962) has shown that thyroxine increases sodium intake into the yeast cell and causes a decrease in potassium leakage.

Work in mammals, both <u>in vivo</u> and <u>in vitro</u> has, however, been performed recently. Aschkenasy (1963) has shown an increase in rat red cell uptake of radioactive iron under the influence of thyroxine and Ermans (1960), working on both rat and human erythrocytes, showed by measuring phosphate turnover, that the fraction of acid-soluble phosphate replaced per minute is increased in the hyperthyroid condition and decreased in the myxoedematous state. Menozzi and Gatto (1961), again working on rats, but using epididymal adipose tissue, showed that thyroxine increased

the sodium content of the cells. Thyroid administration to dogs, rats and frogs reduced the gastric secretion of all these animals, as well as affecting the metabolism of the tissue. The thyroid hormones reduce the ability of the gastric mucosa to mobilize secretory energy in response to a stimulus, but this effect is apparently not correlated directly with their calorigenic properties (Nasset and Goldsmith, 1961). In rats made hyperthyroid by treatment with thyroid inhibitor drugs there is an increase in the total amount of sodium and potassium in the urine (Stephan, Jahn and Maetz, 1959 a,b), which indicates that there is decreased tubular reabsorption of these ions in this condition and injection of thyroxine causes the resumption of normal resorptive processes. A similar situation was found by Fregley, Brimhall and Galindo (1962) where rats treated with propylthiouracil lost more sodium than the controls. The role of the thyroid gland in the osmoregulation of fish has attracted much attention (see Fontaine, 1956) and although changes in thyroid activity have been shown to occur in migrating animals, there is little definite evidence of a role in ionic regulation by the thyroid within the teleosts. Koch and Heuts (1942) moted that feeding of thyroid to goldfish caused a retention of chloride when transferred from sea to fresh water and Hickman (1959) working on the flounder showed a definite correlation between thyroid activity and osmoregulation. The only work which appears to have been performed investigating the effect of analogues on ion movement is that of Hakkila, Siltanen, Miettinen and Karki (1962) who found that TRIAC caused an increase in the outward exchange of potassium and in the net loss of

potassium from guinea-pig heart muscle. This effect was transitory, having a duration of two minutes.

In the Amphibia little is known of the control of thyroxine on ion movements across either the skin or urinary bladder. Koefoed-Johnsen and Ussing (1949) whilst studying the effects of corticotropin on active salt uptake in the axolotl, made a few observations on three animals injected with thyroxine, these animals showed an increase in salt uptake as compared with the controls Jørgensen (1949) showed that during moulting in toads, a process in which thyroxine is known to have an important role, the sodium influx, measured isotopically, is increased indicating, in the light of studies on Amphibian skin since that time, that active sodium transport is increased. It is, therefore, evident that a more complete and detailed study of the effects of thyroidal hormones on ionic movements through the Amphibian skin and bladder tissues, particularly as they have been ably shown to be suited to such studies, would add to our knowledge of the action of the thyroidal hormones at the membrane level and also to knowledge of the effects of the hormones in the Amphibia.

Results obtained previously using both sodium-free incubation media (II, C, 4) and specific inhibitors of cellular enzyme processes (II, C, 3) indicated that both thyroxine and triiodothyronine were, in addition to increasing water permeability, increasing the metabolism of the toad bladder (IV, C), particularly that part of the metabolism directed towards sodium transport. It was therefore felt that a more concise study of thyroidal hormone effects on such ion movements could be of value in understanding the

effects of these hormones on permeability processes and on the underlying metabolic control of these processes.

B. MATERIALS AND METHODS

1. Animals

Mature Bufo bufo of both sexes, obtained either from a commercial source (L. Haigh & Co. Surrey) or collected locally, were used in this study. The animals from the commercial source were not used for at least two days after arrival and were not fed during the short period before use. These animals were never kept more than fourteen days after arrival. Animals collected locally were fed three times a week on meal worms and flies when kept for long periods, this diet being supplemented by force feeding with a liver-egg mixture (homogenised in a Waring blender) at least every two weeks. The animals retained their ability to self feed after this process. With this procedure, the animals, which were all kept at 12°C, were maintained in good health. The obviously more healthy animals were, however, selected for these studies.

2. Salines

The saline incubation medium used was as follows:-

1. NaCl 6.5 g./l., KCl 0.2 g./l., $CaCl_2$ 0.2 g./l., $MgSO_4$ 0.1 g./l., $NaHCO_3$ 1.5 g./l., Na_2HPO_4 0.1 g./l. and glucose 0.75 g./l. which was adjusted to a pH of 7.8 where necessary with the appropriate NaOH or HCl solutions. The osmotic activity was 260 milliosmoles.

In certain studies the following chloride-free saline was used. 1l. Na_2SO_4 5.0 g./l., K_2SO_4 0.2 g./l., $CaCl_2$ 0.2 g./l., $MgSO_4$ 0.1 g./l., $NaHCO_3$ 1.5 g./l., Na_2HPO_4 0.1 g./l., and glucose 0.75 g./l., which also was adjusted to pH 7.8 where necessary. The osmotic activity was adjusted to 270 milliosmoles using mannitol.

Both saline were stored in stoppered vessels at room temperature and not kept for more than three days after being made up. Sodium penicillin was added to the salines in later studies, particularly those using toad skin, at a concentration of 150 units/ml.

3. Apparatus

Three types of apparatus were used in these studies and two of them are depicted in Figs. 9 and 10. Both types I and II are based upon that described by Ussing and Zerahn (1951). Type III being a modification of the technique for a different type of experiment. All types of apparatus were cut from perspex.

The first apparatus consisted of two perspex chambers (see Fig. 9) area of the chamber opening 0.56 cm^2, having volumes, determined by weighing with mercury, of the right hand side 2.79 ml. and the left hand side 2.84 ml. Both chambers were siliconed on their inner surfaces after being smoothed and highly polished. All connecting tubes were of polythene tubing and these were sealed into the chambers using a polystyrene cement. The volume of each chamber, connecting tubes and the glass reservoir was 15 ml, each reservoir holding approximately 8 ml. of fluid. The aeration system also served as a lift pump and circulated the fluid

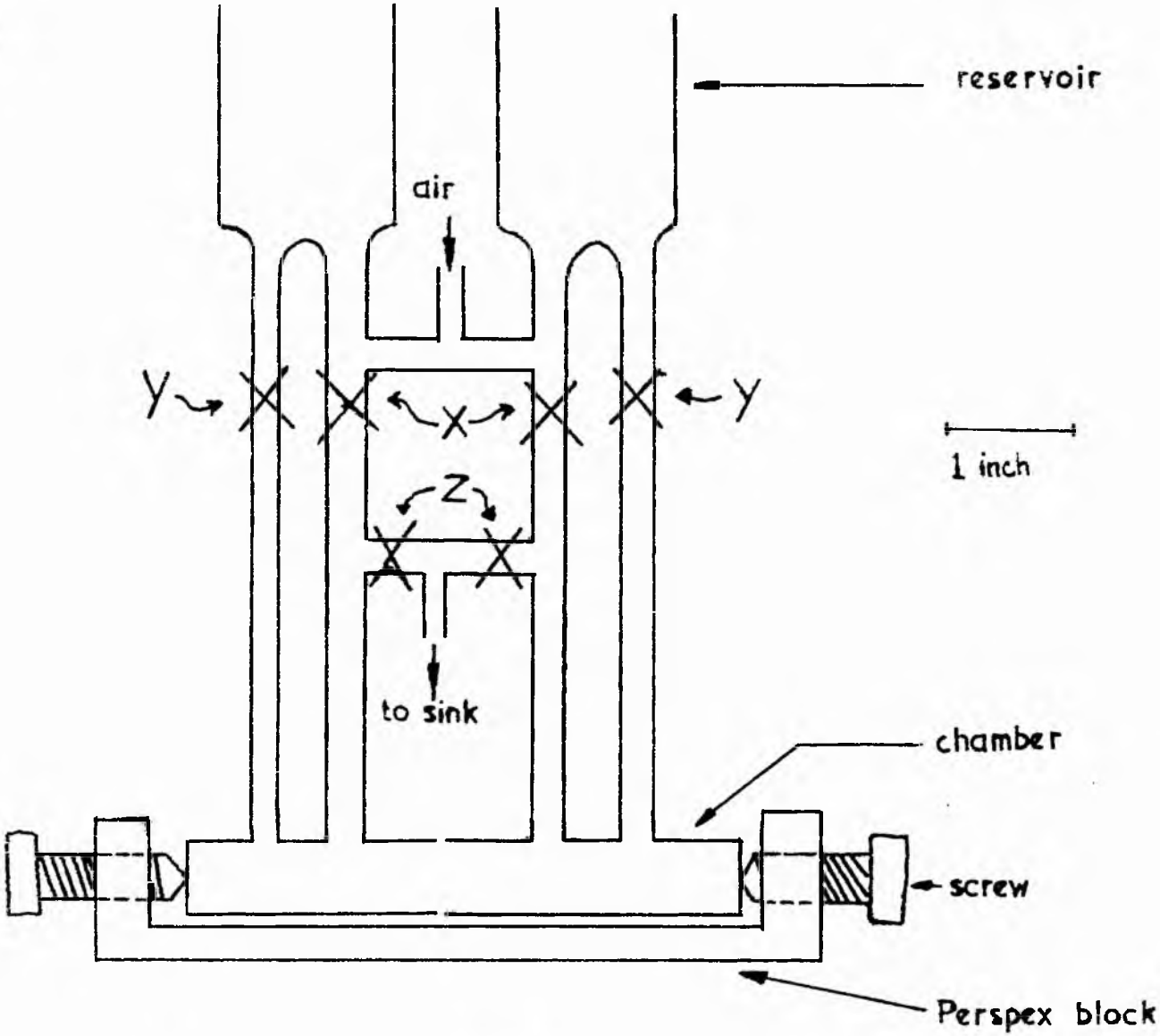
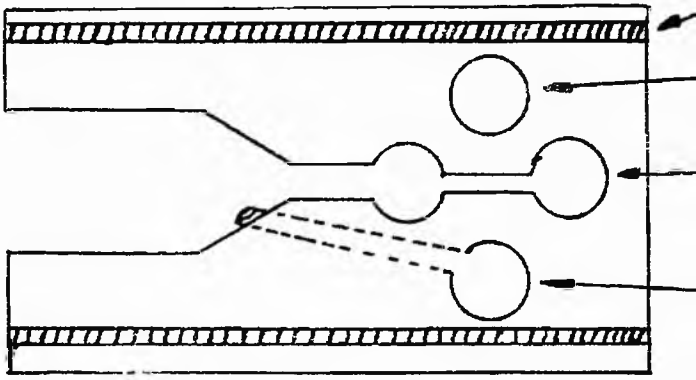


Fig. 9. Diagram of apparatus I for measuring electrical characteristics of Amphibian membranes.

PLAN



SAGITTAL SECTION

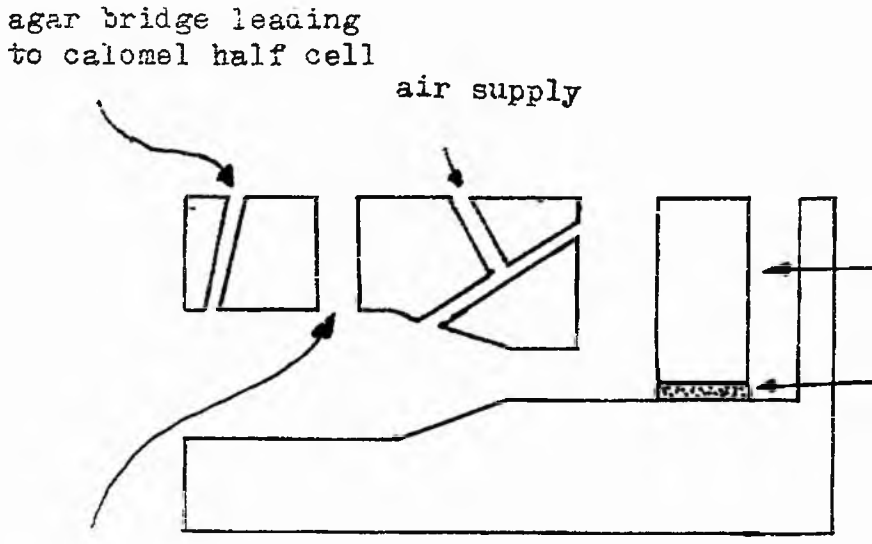
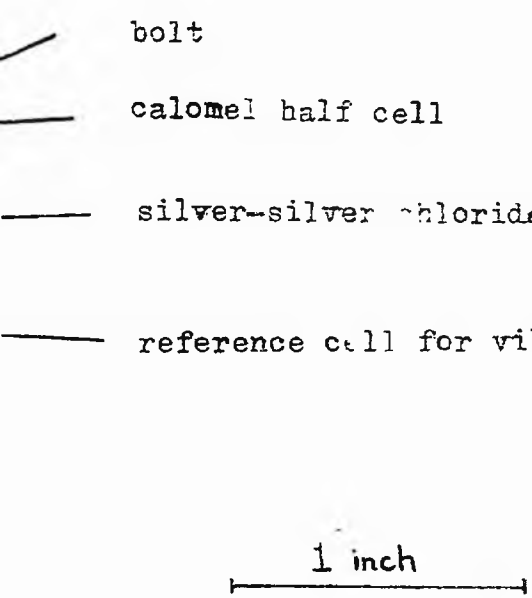


Fig. 10. Diagram of apparatus II.



to show the position of the half cells and bridges.

in the chambers; the circulation was checked periodically using fine carmine particles in the water. Taps were placed in the connecting tubes which enabled the circulation to be stopped and this was achieved by means of a tap (X) immediately below the air supply inlet. A tap was also placed on the inflow tube to the chamber (Y). A separate system was incorporated by which it was possible to change the fluid on one side of the membrane without altering the hydrostatic pressure exerted on the other side. This consisted of a gravity emptying device let into the outflow tube from the chamber with it's own tap (Z). With X closed, Y open and Z open the pressure of fluid in the reservoir pushed fluid from the chamber and out of the emptying tubes via Z. By maintaining a steady inflow of saline it was possible to completely change the fluid on one side whilst maintaining the circulation on the other. It was equally possible to close off X, Y and Z on one side and open them on the other side, thereby draining one chamber. If the system was completely air and water tight then the fluid on the closed side did not tend to stretch the membrane when fluid was drained from the opposite side.

The two chambers were held together in a shallow perspex dish by a screw at either end. These screws were centred and fitted into small conical depressions in the centre of each outer end of the two chambers. Four bridges, which consisted of fine polythene tubing, internal diameter 0.75 mm, filled with 3% (w/v) agar made up in the saline medium which was used for a particular experiment, were inserted into the chambers as shown in Fig. II.

They were pulled narrower at the tips to reduce ion exchange with the medium and greased before being pushed into the chambers to make the joints water tight. The two bridges at the open ends of the chambers were placed so that the tips were at the dead centre of the chamber at that point. With a membrane in position they were approximately 3 mm. apart. The outer ends of these bridges dipped into calomel half cells and these in turn were connected to the respective poles of a Pye Dynacap millivoltmeter, with a 0-20 m extension meter built into it, or a Radiometer pH meter 4.

The other two bridges were at the closed (or outer) ends of the chambers, as far away from the membrane as possible, but still having their inner tips in the centre of the chamber at that point. The outer ends of each bridge dipped into two separate tubes containing saturated potassium chloride, which was further saturated with silver chloride. This solution was connected, via coils of chlorided silver wire which were immersed in the solution, to a d.c. microameter and the external voltage supply (see Fig. 11.).

The second apparatus was cut from a single block of perspex. Each half block had a chamber cut into it and the half cells and bridges were all contained in the one block (see Fig. 10). The calomel half cells were placed to one side of the block and connected to the chamber by means of an external bridge, which passed through a hole in the block so that the tip of the bridge was at the dead centre of the chamber at its open end. The calomel cells were connected via platinum wire sealed into the block with 'Araldite' (Ciba Ltd.) and ordinary lead wire to either a Pye Dynacap

millivoltmeter, with a 0-20 mV extension meter, or a Radiometer pH meter 4. The silver-silver chloride cells were connected to the chambers via the internal 3% (w/v) agar bridge, injected with a syringe, and to the external circuit via the coils of chlorided silver wire immersed in the fluid. Aeration and circulation were performed by the inter-connecting tubes shown in Fig. 10.

This apparatus was self aligning, as four bolts were placed through both blocks, one at each corner, but were permanently fixed only in one block, the other block being free to slide along the bolts. It was secured tightly against the other block by means of four retaining nuts. This latter apparatus had the advantage of being more compact. The internal area exposed was 1 cm^2; the volumes of the chambers, determined by weighing with mercury, were right hand side 2.95 ml. and left hand side 2.99ml.

The chamber areas were siliconed on their inner surfaces after being smoothed and highly polished. From an earlier model it was found to be an advantage if the tubes to be used as internal bridges were left unpolished after drilling, as the slight irregularities in the surface offered a grip for the agar. The half cells were sealed with small perspex plugs with a central hole in them to allow connecting wires to pass through.

All soldered joints in both sets of apparatus were thinly covered with 'Araldite' to prevent any unwanted electrical side effects if these came into contact with saline media. The silver wire was chlorided by electrolysis, depositing silver chloride onto clean silver wire by passing a very low current through a potassium

chloride-silver chloride solution with the silver wire coils as the anode. They were usually chlorided overnight at this very slow rate in the dark and gave an even deposition of chloride. The tubes containing the wire coils were blackened to prevent decomposition of the silver chloride to silver and chloride ions, which occurs when it is exposed to light.

The third apparatus consisted of a single chamber cut into a block of perspex, area exposed, 0.442 cm^2, and internal volume 1 ml and a number of these were used. Each of the blocks had a plate over the open end, with a hole, exactly that of the area of the chamber opening, cut into it. The plate was held into place on the open end of the chamber using four nylon screws and between this plate and the chamber a membrane could be placed. Such chambers could then be placed in larger containers. Aeration was performed using a bent hypodermic needle inserted down a tube drilled near the open end. This tube was also used to hold an agar bridge during potential measurements. The potential was monitored, but not short-circuit current, using this apparatus by two agar filled (3% (w/v)) bridges placed close to the membrane on either side.

4. General Procedure

The pithed animal was pinned out and either a piece of ventral abdominal skin was removed, or the animal opened to expose the abdominal contents and the bladder removed. The skin or bladder was then placed in a petri dish containing saline at 25°C. If the bladder was distended it was found that less physical damage

ensued if it was cut slightly at the base to allow drainage of urine. The bladders were cut out of the abdomen by freeing the mesentery attaching them to the body wall and after removal the bilobed bladders were separated into the two lobes. One lobe was then opened at the neck formed by separating it from the other half, using fine but dulled forceps, and cut open to form a flat sheet. This was then placed over one of the open ends of the chambers. Using apparatus II it was essential to have both the opposing surfaces absolutely dry, otherwise the half bladder or skin slipped when placed onto the surface. Both membranes were wiped on a clean sheet of glass to partially dry them before placing in the apparatus.

The piece of skin or half bladders, after removal from the animal, were mounted in either apparatus at A (see Fig. 11) between the two separate halves of the chamber. The chambers were then clamped together either by the large screw at each end (apparatus I) or by the four retaining nuts (apparatus II), thus making the systems air and water tight. Apparatus I was filled by pipetting 15 mls of saline onto each side of the membrane, and apparatus II by placing 2.75 ml. in either chamber. Air bubbles were eliminated and the circulation system begun. Membrane potential was measured on the electronic apparatus described (E) via the two bridges (A_1, A_1,) placed close to the membrane. Ussing and Zerahn (1951) have shown that when an external E.M.F. is applied to a membrane, bathed on each side by the same medium, and the membrane potential is adjusted to zero, the membrane is short-

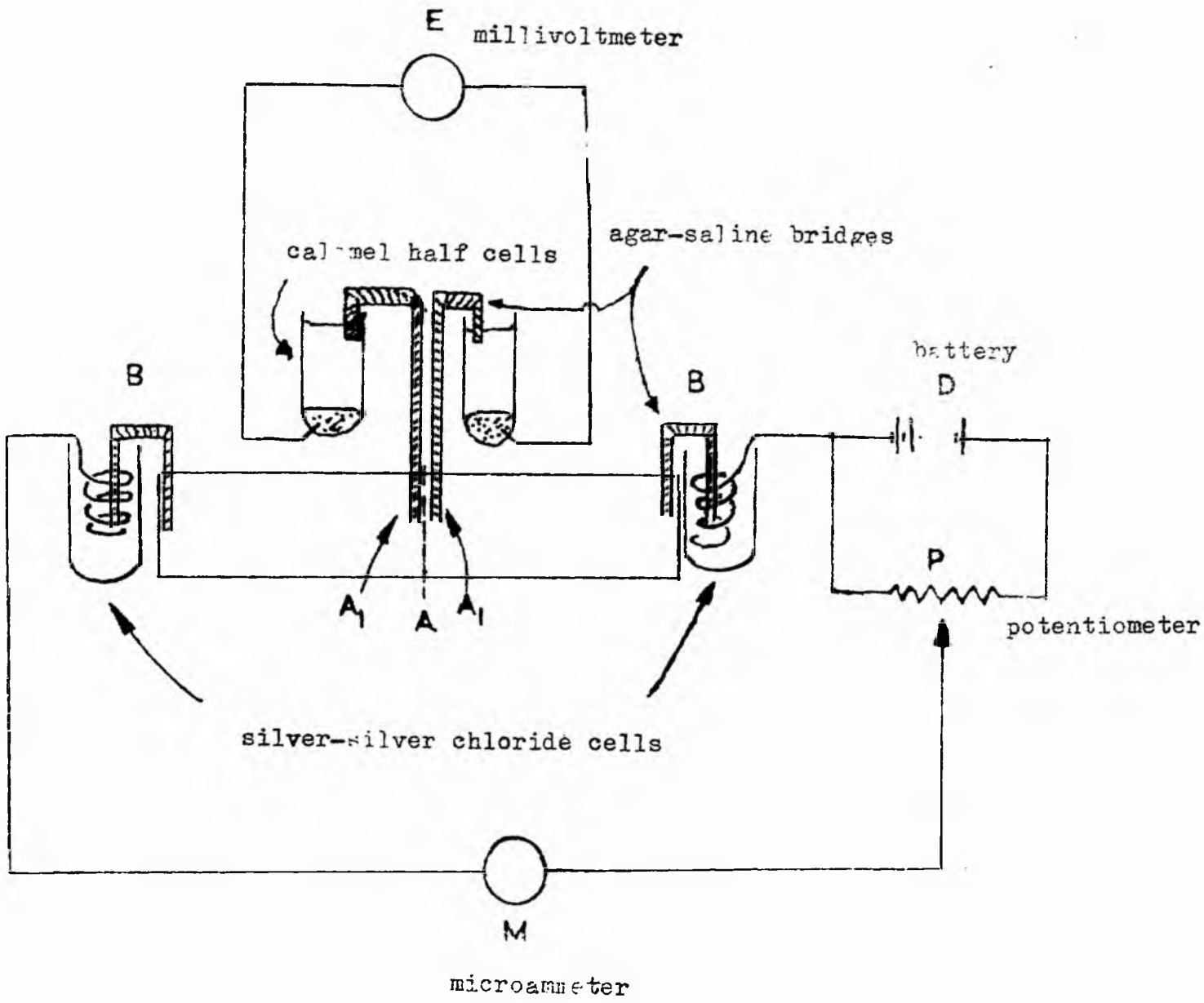


Fig. 11. Circuit diagram for both apparatus I and II, showing the bridge arrangement as used in apparatus I and II.

circuited; the current measured in the external circuit being equal to the active ion transport through the membrane. Thus an external E.M.F. could be applied to the membrane by adjusting the voltage from the dry battery, D, using the potentiometer, P, (Fig. 11). This E.M.F. being transferred to the membrane through the silver-silver chloride cells and the bridges (B, B,) at the outer ends of the two chambers. The current flowing in the external circuit was then measured on the d.c. microammeter, M. The potential or short-circuit current was usually monitored continually, so that unexpected changes in current strength could be recorded, should they occur, although readings at five minute intervals usually sufficed for a measurement of the amount of electricity crossing the membrane. The junction potential, created as a result of exchange of ions between the bridges and the media used, was measured both before and after each experiment and if any such potential was found this was subtracted from those measurements made during the experiment. The membrane was usually allowed one hour to equilibrate, during which time the saline medium was changed three times to thoroughly wash the membrane. The procedure after this time varied according to the experiment performed, but most of the experiments were performed in conjunction with measurements of oxygen consumption (see IV). In these experiments the same technique applied. After the one hour equilibration period the inlets and outlets of apparatus I were quickly clamped with artery clamps. In apparatus II, the holes in the block were sealed with greased, tightly fitting perspex plugs, one of which,

on each side, had a hole in it to allow excess saline to come out. This was filled with plasticine when the excess fluid had been dried off. After forty-five or sixty minutes the saline was then allowed to regain the oxygen concentration of the air at that particular temperature and the hormones were added in either of the two ways described (III, B, 11, 12). The chambers were clamped at this time as well and the parameters monitored for periods of one hour or more. The temperature of the saline during the experiments was 20-25°C. Pieces of bladder or skin were removed from the apparatus at the end of an experiment, trimmed to the exact internal area exposed to the saline, which is readily done since the surrounding tissue is squashed, and dried to constant weight at 105°C.

During isotopic measurements (see III, B, 8) the inlets and outlets were not clamped off, neither was the oxygen consumption determined.

5. Circuit

The same basic circuit was used in both sets of apparatus for measuring short-circuit current (see Fig. 11). A 48 volt dry battery was connected to the two stationery poles of a 2 megohm slide-wire potentiometer (accuracy ±10%) with the sliding pole connected to a 0-50 μamp d.c. microammeter (Fe and NFe). The other pole of the microammeter was connected to the silver wire coil on one side of the chamber, whilst the other silver wire coil was connected to one of the stationary poles of the potentiometer. The arrangement was such that whatever orientation of the membrane in the apparatus used it was possible to interchange the polarities

of the circuit and thus continue to measure the potential and short-circuit current.

6. Precautions

With apparatus I, it is essential that there is no hydrostatic pressure across the membrane, therefore the glass reservoirs must be at the same level and when each contains 15 ml. the height of th meniscus from the chamber must be the same in each case. Constant checks must be made on the fluid level in the calomel half cells and the tubes containing the silver wire coils. It was found that the saturated potassium chloride tended to 'creep' over the edges of the tubes and this was prevented by placing a smear of grease around the top of the tubes, where the rubber bung fitted in. Grooves were cut in the bung for the appropriate polythene agar bridge and wire connections to pass into the tubes and these were also lightly greased. The bridges were frequently checked and trimmed to expose the agar if the agar had contracted into the tube. The apparatus was left filled with saline when not in use for more than thirty minutes. With apparatus II it was found that little attention was required to the bridges as long as, after experiments had been completed, the chambers were filled with saline solution to keep the bridges moist. Here, as in apparatus I, excessive drying of the half cells and 'creep' of potassium chloride was prevented by the application of a smear of grease around the top of the tube. Whenever any of the tubes was found to have dried, a solution of saturated potassium chloride was always used to restore it to the original level. Constant checks were also necessary on the chlorided silver wire coils and whenever they were found to

show 'pitting' or unevenness of distribution of the chloride, they were cleaned and replated. No difficulty was found with mounting of either tissue in either apparatus. It was found to be advantageous, however, if, when mounting the tissue, the surface of one chamber was thoroughly dried and one surface, preferably the mucosa of the membrane was drained of excess moisture on a clean sheet of glass. This enabled the membrane to remain in place more readily. The time taken from draining the tissue to adding the saline on either side, in any apparatus used, was less than 30 seconds, thus it is unlikely that the tissue would dry at all during this time, therefore as little physical damage as possible was done to the tissue.

Care must be taken over the position of the bridges in the chambers. It is essential to have the tips of all the bridges in a straight line drawn through the centre of the chambers. The potential measuring bridges must be very close to the membrane, and equally the short-circuit current bridges as far away as possible, but equidistant from the membrane. The latter is done in order to obtain an even current density over the membrane. Equivalent distances of the respective pairs of bridges from the membrane is important otherwise false readings may be obtained, and it is essential to have a firm support for the bridges to prevent any movement which may take place.

7. Thyroxine: Triiodothyronine mixture

Following the work on the effect of a mixture of these hormone on water permeability of the isolated bladder (II, C, 7) experiment were performed to investigate the effects of such a mixture on the

potential and short-circuit current of the bladder.

8. Isotonic flux measurements

Flux measurements were made in both directions through the toad membranes, particularly of the toad bladder.

(i) Sodium

These experiments were performed using apparatus I and flux ratios were determined for both skin and bladder of the toad using Na^{24} (Radiochemical Centre, Amersham) with the membrane under short-circuited conditions, the short-circuit current values being noted. The mucosal to serosal and serosal to mucosal fluxes were determined in separate experiments at room temperature (22-25°C) and were all determined using one batch of Na^{24}. Since Na^{24} has a half-life of some 15 hours, it was necessary to add an increasing amount of the isotope to the bathing fluid during the four days of the experiments. Initially, only 0.1 ml. of the 'hot' solution was added, but in order to maintain a constant amount of Na^{24} added to the solutions it was necessary on the fourth day to add approximately 1.5 ml. of the 'hot' solution. In every experiment roughly 25 μc of the isotope were added to the bathing medium in which it was required, which gave a count rate of approximately 500,000 counts/ml. of solution/100 seconds. Initial experiments using sodium Na^{22} indicated that such high activities were necessary in order to detect any movement of sodium through the membranes. After the equilibration and washing period of one hour, the isotope was added to one side of the short-circuited membrane and allowed to equilibrate for thirty minutes. Since thirty minutes were allowed for equilibration of the isotope with the membrane it is

unlikely that any rapid tissue-fluid exchange would effect the data, for Hoshiko and Ussing (1960) have shown that the half-time of sodium exchange in the toad bladder is four minutes. Thus equilibration of the membrane with the Na^{24} would appear to take place rapidly.

Control periods of 40 minutes were used, samples being taken at 10 minute intervals during this time. After addition of a concentrated hormone solution, of known volume, samples were taken from both sides of the membrane at two minute intervals for 10 minutes and then at five minute intervals for the rest of the 60 minute treated period. 0.2 ml. samples were taken at each of these times using a constriction pipette, of which several were in use, and each was carefully washed out and air-dried before subsequent use. The samples were transferred onto planchettes together with a little liquid detergent (the latter to aid in spreading the fluid and dried under an infra-red lamp. The samples were then counted with a thin-end window Gieger-Miller tube in conjunction with an automatic Ecko scaler. The time of addition of the isotope, time of beginning the experiment, time of removal of the sample from the bathing solution and the time of counting were all carefully noted. Suitable corrections for decay, (derived from graphs plotted from the relationship $\log 100\ N/No - 2 - \frac{(t \log 2)}{t_{\frac{1}{2}}}$, Francis, Mulligan and Wormall, 1959), background effects and the effect of the removal of samples from the bathing medium, as well as for the addition of the hormone solutions were all applied to the resulting data. The hormone was always added on the serosal surface of the membrane as a concentrated solution.

(11) Chloride

The flux determinations were performed on the short-circuited bladder in apparatus II. The separate fluxes, mucosal to serosal and vice versa, were again performed in separate experiments. A stock solution of saline containing radio-active Cl^{36} (1.4 μc Cl^{36}/ 10 ml.) (Radio-chemical Centre, Amersham) was made up and used in each experiment. The bladder was allowed to equilibrate for 45 minutes during which time the saline was changed three times. The labelled solution was then substituted for the normal saline on the appropriate side of the membrane, care being taken to empty the chamber completely of normal saline and left for 45 minutes in the short-circuited condition. 0.1 ml. samples were then taken by means of constriction pipettes at 0, 30 and 60 minutes from both sides of the membrane during the control period. Thyroxine was added in 0.1 ml. to make a final concentration in the serosal chamber of 10^{-6}M and a similar amount of ordinary saline was added to the other side. A sample was taken at this time and was the zero time sample, further samples of the fluids bathing both sides of the membrane were taken at 30, 60 and 90 minutes after addition of the hormone.

Samples were transferred onto planchettes, on which a spreading agent had been placed, dried and counted using an end window Geiger-Müller tube. Since Cl^{36} has a long half-life (3 x 10^5years) no decay corrections were applied. Corrections for removal of samples, and addition of fluids were applied, as were background corrections.

(iii) Phosphate

Flux determinations of P^{32} (as orthophosphate from the Radiochemical Centre, Amersham) were made on the short-circuited toad bladder in apparatus II. The fluxes from mucosal to serosal surfaces and vice versa, were determined in separate experiments. This isotope has a half life of 14.3 days and all the experiments were performed within 2 days of arrival of the isotope. A stock solution was made up having an activity of 30 μc/10ml. saline and during two days this decayed to approximately 90% of its original activity.

After an equilibration period of 45 minutes, during which time the saline on both sides of short-circuited membrane was changed three times, the saline media were then removed and replaced with normal saline on one side and the labelled solution on the other. This was then left for 45 minutes for the membrane to equilibrate with the isotope. After this time 0.1 ml. samples were taken, by means of constriction pipettes, at 0, 30 and 60 minutes from both sides of the bladder during the control period. Concentrated thyroxine solution was then added in 0.1 ml. to the serosal surface to give a final concentration of 10^{-6}M and a similar quantity of normal saline was added to the mucosal surface. A sample was then taken at this time and at 30, 60 and 90 minutes after this time.

Samples were transferred to planchettes, to which a spreading agent had been previously added, dried and counted using a thin end-window Geiger-Muller tube. Corrections for decay were applied to the data, as were corrections for removal of samples and

addition of fluids. The background count rate was also subtracted from the data.

(iv) Urea

Flux determinations of urea movement, using uniformly labelled C^{14} urea (Radiochemical Centre, Amersham) were made on the short-circuited toad bladder in apparatus II. The fluxes in each direction across the membrane were determined in separate experiments. A stock solution of the isotope was made having an activit of 20 μc/100 ml. of saline which also contained 150 units/ml. sodiu penicillin. This was stored at 4°C. and enough of this solution for an experiment was brought up to 20-22°C. when required.

After an equilibration period of 45 minutes, during which time the saline was changed three times, the urea solution was added to the appropriate side of the short-circuited membrane and a similar molar quantity of non-labelled urea was added to the othe bathing media. This was left for 45 minutes and a sample was taken at this zero time of the experiment; further samples were taken at 30 and 60 minutes during the control period. The thyroxine was added in 0.1 ml. to the serosal surface as a concentrated solution, yielding a final concentration in the chamber of 10^{-6}M. A similar quantity of normal saline was added to the mucosal surface and samples were taken of both fluids. Samples were then taken at 30 minute intervals after this time for 90 minutes.

Samples of 0.1 ml. were taken from the fluids and counted in an Ecko liquid scintillation counter. The samples were placed in a phosphor having the following composition, 2:5 - diphenyloxazole 4 g./l., 1:4 - di - (2-(5-diphenyloxazole)) benzene 100 mg./l.,

naphthalene 50 g./l. made up in 'Analar' grade Xylene (B.D.H.). It has been shown that the effect of adding quenching agents, such as water, can be reduced by a xylene; naphthalene mixture (Kallman and Furst, 1958; Brown and Badman, 1961) and this phosphor, even after the addition of 0.1 ml. sample, was found to have an efficiency of roughly 60%.

Corrections were applied for this efficiency factor, for the background count rate and the effect of removal of samples and addition of fluids.

(v) Calculations

Sodium flux values, $^{M}M_{S}$ and $^{S}M_{M}$, were calculated from the following equation:-

$$\frac{dC_i^*}{dt} = {}^{S}M_{M} \cdot \frac{A}{v} \cdot \frac{C_o^*}{C_o}$$

where $\frac{dC_i^*}{dt}$ is the increase of specific activity per hour of the mucosal fluid (counts/ml./unit time), A is the area exposed in cm^2, v is the volume of the outside solution in ml., C_o is the serosal concentration of sodium in μequiv./ml. and C_o^* is the specific activity of the serosal radioactive saline in counts per ml. The back flux of isotope was neglected. The same equation was used for calculations of M_{in}, the mucosal to serosal flux, substituting the different values obtained. The flux was therefore obtained in μequivalents /cm^2/unit time.

From the results of the measurement of Cl^{36}, P^{32} and C^{14} labelled urea movement it is possible to calculate a permeability coefficient, K_{trans}, which is defined as the amount of a given substance crossing 1 sq. cm. of membrane surface per second under a driving force of unit concentration gradient and has the

dimension of centimetres per second, thus

$$K_{trans} = \frac{\text{increase in counts on the unlabelled side}}{\text{concentration of counts on labelled side (specific activity) x area x time.}}$$

(Maffly, Hays, Landin and Leaf, 1960). The results of the fluxes are expressed in these terms for comparison with other work. The flux values are calculated from the 0-60 minute transfer values, but no difference is seen if the values of 0-30, 30-60 and 60-90 minute values are compared.

9. Tissue content of sodium and potassium.

These experiments were performed using apparatus III. Paired half-bladders from the same animal were set up in these sets of apparatus, one half being a control and the other being a treate tissue. The sodium and potassium content was determined in separate experiments. Twenty four half bladders were placed with the mucosal surface of the bladder facing the inside of the chamber for the sodium determinations; whilst a similar number of half bladders were set up with the serosal surface facing the inside of the chamber for the potassium determinations. They were allowed to equilibrate for 1 hour, during which time the saline was changed twice. After this hour, time zero, 1 ml. of a stock solution containing Na^{22} was applied to the mucosal surface of each paired half bladder; and a similar volume of K^{42} was applied to the serosal surface of the other paired half bladders. The stock Na^{22} solution had a concentration of 15 μc/100 ml. and the K^{42} solution of 50 μc/100 ml. One half of each paired half bladder was thus treated, whilst the other half bladder received a 10^{-6}M thyroxine solution in addition to isotope. Experiments were thus performed

in which thyroxine was applied either on the mucosal surface, serosal surface or on both sides at the same time and in each case, whether for Na^{22} or K^{42} content determinations, the same regime as described above was followed. At 15 minutes after addition of the isotope (or isotope plus hormone) three control half bladders and their corresponding paired half bladders were removed from the sets of apparatus, washed once in each of three solutions of normal saline, to remove adsorbed solution, and placed as flat sheets onto planchettes. Three half bladders and their paired half bladders were also removed, washed and put onto planchettes at 30, 60 and 90 minutes after the addition of the isotope. Removal of the disc of membrane was achieved with a very fine knife, the membrane being cut out in situ. In addition samples of the serosal bathing fluids were taken, as were samples from the stock solution. Thus it was possible to determine that sodium was in fact being moved and this was supported by measurements of potential. The previously weighed planchettes and bladders were immediately weighed to determine the wet weight of the tissue. They were then dried at 95°C. for 24 hours and then weighed again to determine the tissue dry weight. The radioactivity of the bladders was then determined using a thin end-window Gieger-Muller tube in conjunction with an automatic Ecko scaler.

Thus the results can be expressed as counts per mg. dry wt. or as counts per mg. of tissue water. No corrections were necessary using the Na^{22}, except that of a background correction. The K^{42} however, having a half-life of only 12.5 hours, required decay

corrections in addition to a background correction.

10. Sulphate medium

Saline solution II was used in these experiments and comparisons were made between paired half-bladders, one half in normal saline and the other lobe in sulphate solution, but the effects were limited (see III, C, 4). It was, therfore, decided to use a solution of copper sulphate giving a concentration of 10^{-5}M copper, which was shown by Ussing (1949 b) to affect the potential of frog skin by decreasing the anionic permeability, particularly that of chloride ions. The toad bladder was usually set up as described earlier for apparatus II (II, B, 4) and allowed to equilibrate in the sulphate medium, or after addition of copper sulphate to the mucosal surface. After a 45 minute control period, hormone was added to the serosal surface and potential and/or short-circuit current monitored. Six paired half-bladders were used, one half treated in normal saline, which thus acted as its own control for the normal treatment, and the other half treated with copper on the mucosal surface, with this membrane also acting as its own control to the normal treatment.

11. Analogues

3:5:3';5' - tetraiodothyroacetic acid (TETRAC), 3:5:3' - triiodothyroacetic acid (TRIAC) (Glaxo Labs., Ltd.), 3:5:3':5' - tetraiodothyroformic acid (W 1489, Lot 00125) and 3:5:3':5' - tetraiodothyropropionic acid (W 1524, Lot 8) were dissolved in 0.1 N NaOH before addition to the saline. Equivalent amounts of NaOH were added to the control media.

12. Hormones

L-thyroxine (L. Light and Co. Ltd.) and 3,5,3' - triiodo-L-thyroxine (Glaxo Labs., Ltd.) were dissolved in 0.1N NaOH before addition to the saline medium in which they were required. Equivalent amounts were added to the control media.

C. RESULTS

1. Effect of thyroxine and triiodothyronine on the electrical characteristics of toad skin and bladder.

The short-circuit current recorded from the bladder of the toad, Bufo bufo, using the techniques described varied between 8 and 14 μ amps per sq. cm., from thyroxine treated bladders between 14 and 22 μ amps per sq. cm. after sixty minutes incubation, and from triiodothyronine-treated bladders between 14 and 18 μ amps. per sq. cm. after twenty minutes incubation. The short-circuit current recorded from the untreated skin varied between 25 and 32 μ amps. per sq. cm. and after twenty minutes incubation in 10^{-6}M triiodothyronine between 35 and 42 μ amps. per sq. cm., whilst from skin treated with 10^{-6}M thyroxine values of between 38 and 48 μ amps. per sq. cm. were recorded after sixty minutes incubation. The current recorded from the membranes showed considerable variance between animals. The potentials recorded were of the order of 8 to 12 millivolts for the bladder and 35 to 40 millivolts for the skin; treatment of both bladder and skin with thyroxine and triiodothyronine did not increase the potential more than 15 per cent in either case. The changes in short-circuit current under the influence of these two hormones are summarised in Fig. 12 for the bladder and the skin. Thyroxine, when applied, at 10^{-6}M concentration, to the mucosal surface of the

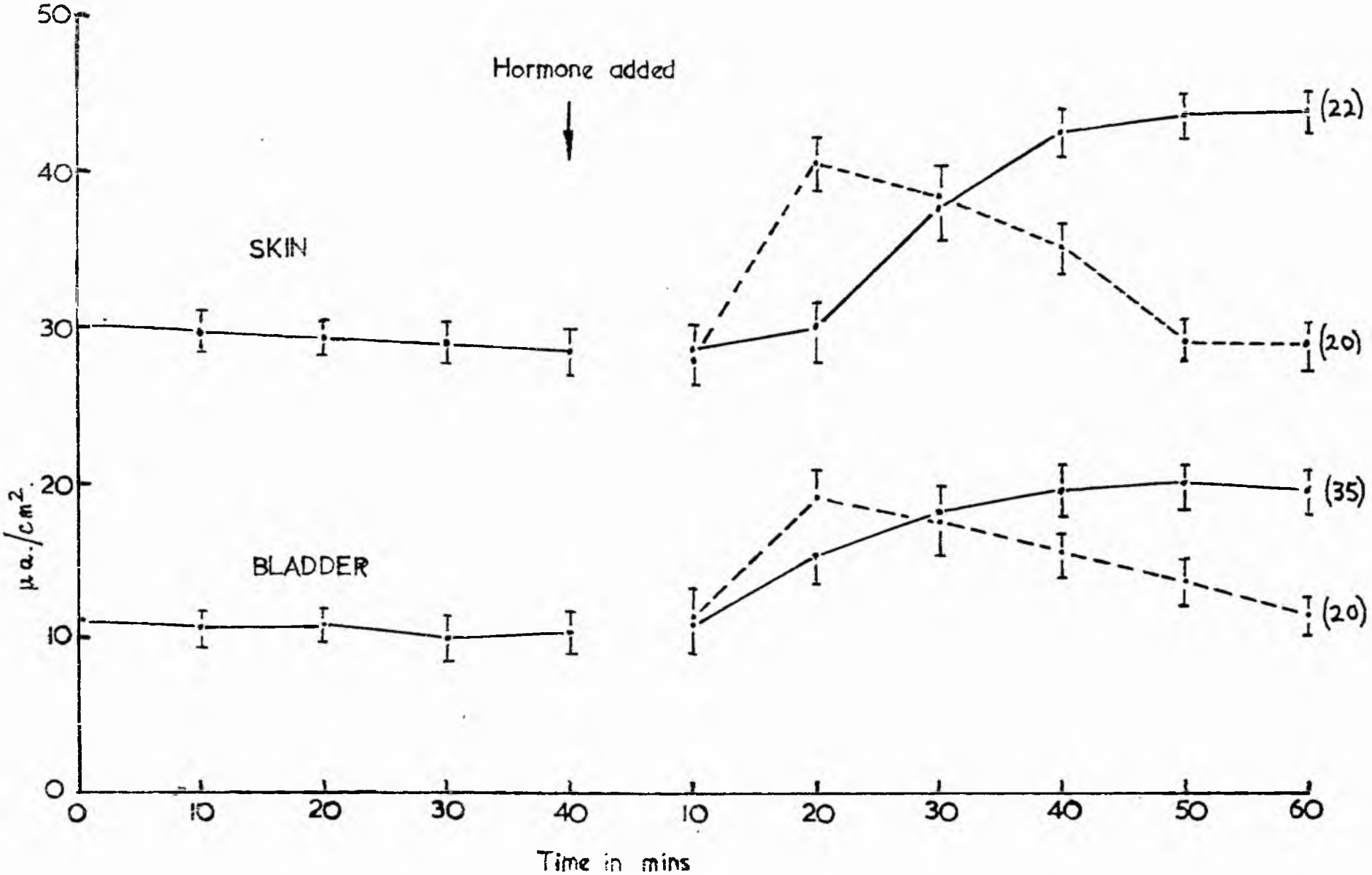


Fig. 12. Effect of 10^{-6} M thyroxine (——) and 10^{-6} M triiodothyronine (- - -) solutions on the short-circuit current of the skin and bladder of the toad.

bladder causes a slight increase in short-circuit current after approximately fifty minutes incubation. The effect is maintained for roughly one hour, the time course showing the beginning of an effect after 30-35 minutes, reaching a maximum at 60-70 minutes and falling slowly to control values after this time. The maximal stimulation is 12-15% above the control values.

The short-circuit current is seen to rise in both membranes within the first few minutes of application of the hormones. The rise is particularly rapid with triiodothyronine which reaches its maximum after only twenty minutes incubation. The maximum with thyroxine, however, is reached only after fifty minutes incubation. This effect was analysed at different doses and the dose response curve for thyroxine and triiodothyronine on both membranes is shown in Fig. 13, the maximal values being used in each case, that is, after fifty minutes in thyroxine and twenty minutes in triiodothyronine.

The short-circuit current is equivalent to a direct measure of the active sodium movement in these membranes (see Leaf, 1955; and III, C, 3,i) and as such can be expressed in microequivalents of sodium per square centimeter per minute (μeq. Na/cm^2./min.), using the relationship 1 μ amp./cm^2./minute equals 6.218×10^{-6} μeq./cm^2./minute, (that is 1 coulomb/1 faraday = eq. Na/sec. therefore 1 μ amp/sec. = 10.36×10^{-6} μeq. Na/sec.). Thus, the bladder increase expressed in these terms is from 0.007 μeq./cm^2./minute to 0.0116 μeq./cm^2./minute under the influence of thyroxine and the skin control value of 0.020 μeq./cm^2./minute rises to 0.031 μeq/cm^2./minute after sixty minutes incubation in 10^{-6}M thyroxine.

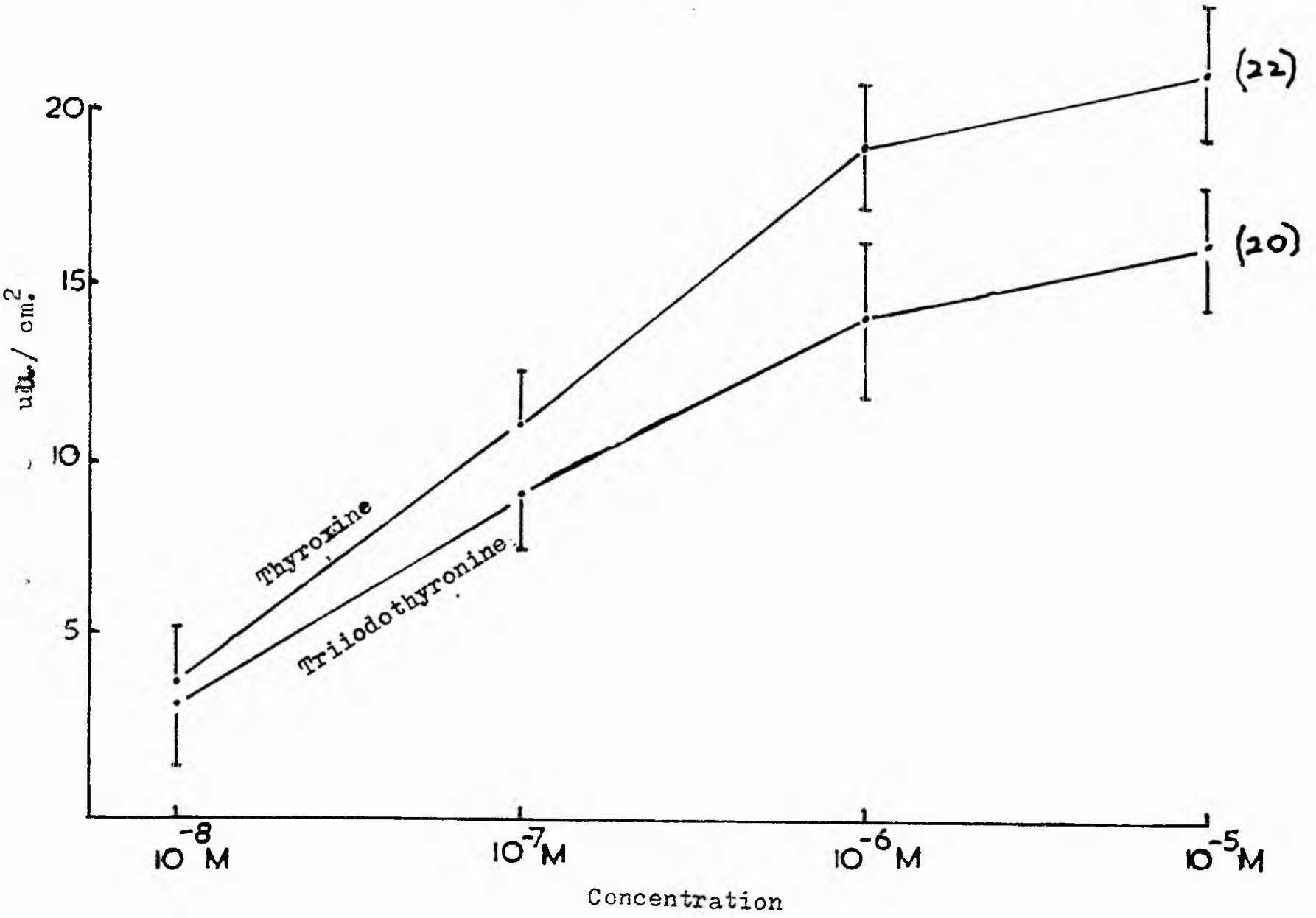


Fig. 13. Effect of different concentrations of thyroxine and triidothyronine on the short-circuit current of the frog bladder. Values are those obtained after one Hour incubation in thyroxine and after twenty minutes in triiodothyronine.

Similar relationships are seen with triiodothyronine on bladder, the control values of 0.007 μeq./cm^2./minute rising to 0.009 μeq./cm^2./minute after twenty minutes and the skin value rises from 0.020 μeq./cm^2./minute to 0.026 μeq./cm^2./minute after twenty minutes incubation at 10^{-6}M.

2. Thyroxine: triiodothyronine response.

Following the work on water permeability of the isolated toad bladder (II, C, 7) where the combined effect of 10^{-6}M thyroxine and 10^{-6}M triiodothyronine was investigated, a similar mixture was found to cause an increase in short-circuit current of the isolated toad bladder. The current rose within the first twenty minutes of application and then showed a slight fall, before increasing to a final maximum which was roughly equal to that in thyroxine alone. The maximum value, however, occurs at roughly forty minutes instead of at fifty minutes which is found with thyroxine alone. There is, therefore, an apparent shift of the curve towards the point of addition of the hormone mixture. The response is shown in Fig. 14 and is biphasic.

3. Isotopic measurements.

(i) Sodium

The tracer studies using Na^{24} are summarised in Table 5, where it is seen that the active sodium transport after sixty minutes incubation in 10^{-6}M thyroxine is increased by fifty-five to sixty per cent, which is in close agreement with the electrical measurements (see Table 6, for comparison of isotopic and electrical measurements confirming work of a similar nature on the equality of the short-

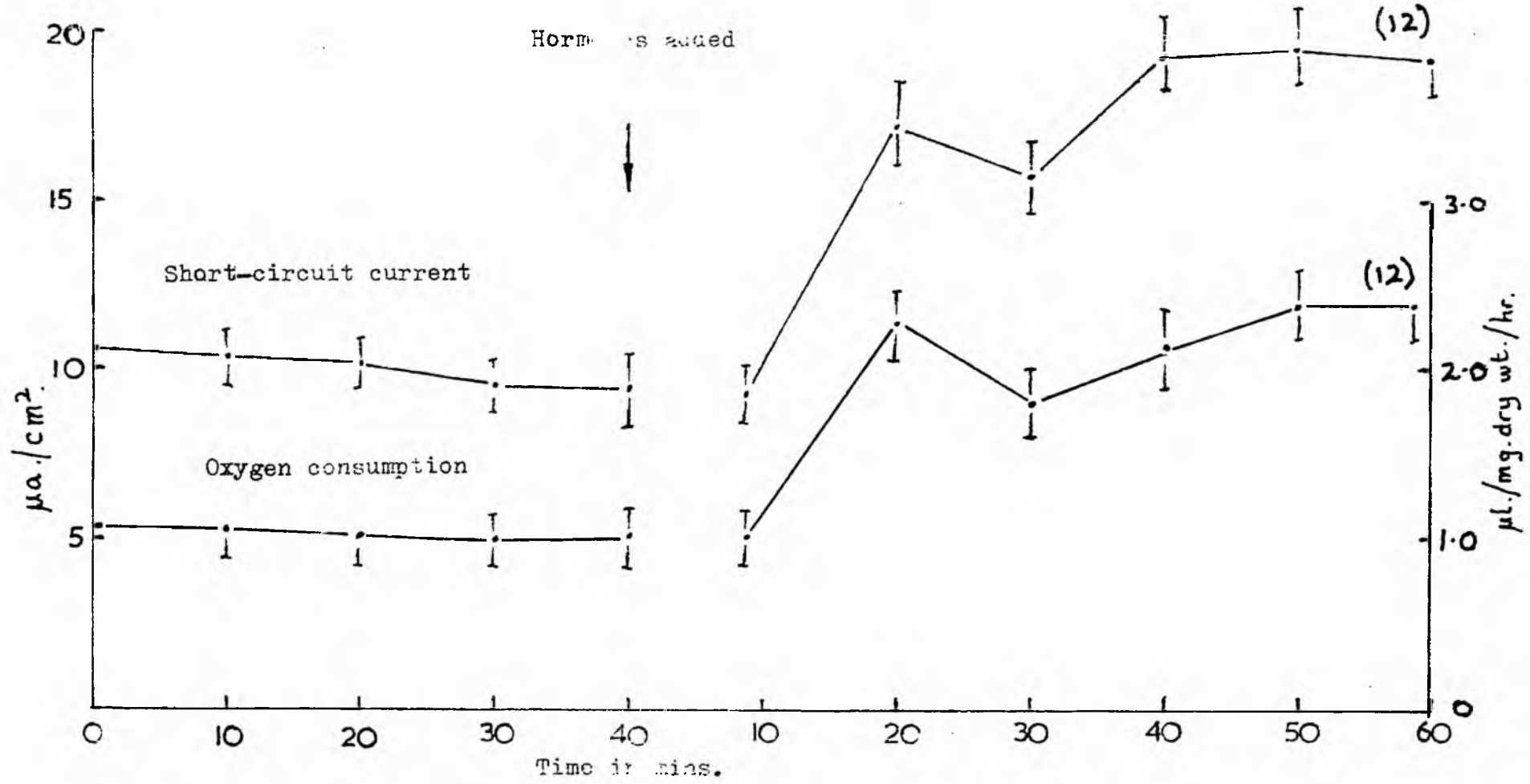


Fig. 14. Effect of a thyroxine: triiodothyronine mixture on short-circuit current and oxygen consumption of the isolated toad bladder. Both hormones added at 10^{-6} M.

Table 5. Effect of 10^{-6}M concentrations of thyroxine on the movement of sodium across the toad bladder and skin using Na^{24}.

Specimen	Mucosal to serosal	Serosal to mucosal	Net flux
Bladder			
Control	0.0209a	0.0128	0.0081±0.0005
Treated	0.0296	0.0171	0.0124±0.0011
Number of periods	6	2	
Skin			
Control	0.0279	0.0080	0.0200±0.0010
Treated	0.0410	0.0100	0.0310±0.0013
Number of periods	5	2	

Values are given as ueq/cm ./minute and are the means (±S.E.).

Table 6. Comparison of Isotopic and electrical measurements of the net flux of sodium across the isolated bladder and skin of the toad.

	Bladder (net flux)		Skin (net flux)	
Specimen	Isotopic	Electrical	Isotopic	Electrical
Control	0.0081±0.0005a	0.0079±0.0005	0.0200±0.0010	0.0192±0.0008
Treated	0.0124±0.0011	0.0118±0.0010	0.0310±0.0013	0.0300±0.0009
Number of periods	8	35	7	22

Values are given as ueo/cm^2/min and are means (±S.E.).

circuit current and active sodium transport (Ussing and Zerahn, 1951; Leaf, 1955; Leaf, Anderson and Page, 1958). It is thus esta blished, using these and the electrical measurements, that the net flux of sodium is increased by both thyroxine and triiodothyronine in near physiological concentrations. This is accounted for mainly by the increased mucosal to serosal movement of sodium, whil there is a slight increase in serosal to mucosal movement. Similarly, in the skin the net flux of sodium is increased in similar manner. It appears, without doubt, by using the criterion of short-circuit current to measure sodium transport and by using radio-active tracer techniques, that both thyroxine and triiodothyronine increase active transport of sodium across the isolated skin and bladder of the toad.

The data obtained from the isotope studies enables one to elucidate on which part of the transport mechanism the hormones are acting. The following equations were devised by Ussing and his co-workers (Ussing and Zerahn, 1951) and made possible calculations of the value of the sodium transport mechanism and other electrical characteristics of the membrane.

It has been shown by Ussing (1949 b) that for a free ion species diffusing through a membrane that

$$\frac{M_{in}}{M_{out}} = \frac{a_o}{a_i} = \frac{C_o}{C_i} \cdot \frac{f_o}{f_i} \cdot e^{\frac{zF}{RT}(\Psi_i - \Psi_o)}$$

Where M_{in} is influx, M_{out} is outflux, a_o, C_o and f_o are electrochemical activity, concentration and activity coefficient of ion in outside solution, a_i, C_i and f_i the corresponding quantities in the inside solution, $\psi_i - \psi_o$ is potential difference across the membrane,

z is the charge of the ion, F is Faradays number, R the gas constant, and T is the absolute temperature.

Thus if the solutions on either side of the membrane are identical:

$$\frac{M_{in}}{M_{out}} = e^{\frac{-zF}{RT}(\psi i - \psi o)}$$

Where the potential difference across the membrane is maintained at zero and the concentration of solution is the same on both sides of the membrane, the transport potential E_{Na} is equal to:

$$E_{Na} = \frac{RT}{zF} \ln \frac{M_{in}}{M_{out}}$$

Also for that part of the D.C. conductivity which is due to the sodium ion (the partial conductivity):

$$K_{Na} = \frac{\Delta}{\frac{RT}{zF} \ln \frac{M_{in}}{M_{out}}}$$

where Δ is the current.

Thus it follows that the resistance to sodium movement (the sodium ion resistance) is

$$R_{Na} = \frac{1}{K_{Na}}$$

The results of these calculations are given in Table 7, where it can be seen that thyroxine has greatly lowered the sodium ion resistance, and therefore has reciprocally increased the sodium partial conductivity with little effect on the actual transport potential of sodium.

(ii) Chloride

In Table 8 it is readily seen from the values of the flux

Table 7. Electromotive force (E_{Na}) of the Na-transporting mechanism, resistance (R_{Na}) to the Na-current, and partial conductivity (k_{Na})[a].

Specimen	E_{Na}:mV	R_{Na}:Ω cm^{-2}	k_{Na}:Ω^{-1}cm^{-2}x10^{-3}
Bladder			
Control	12.94	1232	0.812
Thyroxine	14.61	664	1.506
Difference	1.67	568	0.694
Per cent increase	12.91±1.77	46.1±3.80↓[b]	85.47±4.63
Skin			
Control	33.36	1334	0.749
Thyroxine	37.68	785	1.274
Difference	4.32	549	0.525
Per cent increase	12.97±2.31	41.24±4.01↓[b]	70.09±5.02

a Calculated from the data in Table 1. Values are means (±S.E.).

↓[b] - percent decrease.

of chloride in either direction through the bladder, that movement of chloride is passive, as determined by the flux equation of Ussing, for with no electrical or chemical gradients existing across the membrane the flux ratio is one.

When treated with 10^{-6}M thyroxine, however, a change can be seen in the permeability of the bladder to chloride after one hour The flux rate in each direction is increased by a similar amount thus indicating that thyroxine is causing an effect which allows a freer exchange of chloride ions between the fluids bathing each side of the membrane.

(iii) Phosphorus

The values for the fluxes in each direction across the bladder, given in Table 8, show that this ion moves passively, for the flux ratio, measured in a short-circuited membrane, bathed on each side by similar media, is one. When 10^{-6}M thyroxine is added, the permeability coefficient is increased slightly after one hour, indicating that compared with the smaller chloride molecule, the phosphate ion is less affected by the effect of thyroxine on permeability.

(iv) Urea

The normal permeability coefficient across Bufo bufo bladder is shown to be equal in both directions, indicating the passive nature of the movement of this molecule. After sixty minutes treatment with 10^{-6}M thyroxine the flux rates are increased more than ten-fold (see Table 8). Comparison may be drawn with previous results on water flow take place under the influence of thyroxine. Only water, sodium (in the direction of active trans-

Table 8. Effect of thyroxine (10^{-6}M) on the permeability of some substances across the isolated toad bladder.

Cl^{36}	Control 5.39±0.80	S → M	Treated 13.42±1.21
	Control 6.40±0.92	M → S	Treated 15.97±1.27
P^{32}	Control 3.17±0.74	S → M	Treated 5.41±0.92
	Control 4.14±0.63	M → S	Treated 5.78±0.53
Urea C^{14}	Control 15.36±1.99	S → M	Treated 186.0±18.2
	Control 16.46±L.67	M → S	Treated 201.0±19.73

All values are x 10^{-7} cm/sec S.D. calculated from the 60 min. experimental values. Each directional flux determination is the mean of six half bladders.

port) and urea show these large increases in penetration following hormone treatment.

4. Sulphate medium

The effects of substituting sulphate for chloride in the bathing medium applied to the toad bladder are not as pronounced as those found in frog skin. Paired half bladders were used in this study, one half subjected first to normal saline, then normal saline plus thyroxine and the other half similarly treated, with the chloride in the medium replaced by sulphate. The normal potential difference was 6.3 ± 0.5 mV and in sulphate medium 6.7 ± 0.5 mV, each value being the mean of six half bladders. After one hour treatment with 10^{-6}M thyroxine, a potential of 6.5 ± 0.5mV was measured in normal saline, whereas in sulphate medium the potential rose after one hour to 9.9 ± 0.6 mV (mean ± S.D. of six paired half bladders). It therefore appears that the bladder membrane is almost equally permeable to sulphate ions as it is to chloride ions, in the non-stimulated condition. When treated with thyroxine, however, an increase in potential was obtained in sulphate medium along with a concurrent increase in short-circuit current. A similar increase in short-circuit current was found in normal saline, but as is seen from the results the potential remained almost unaltered.

Treatment with 10^{-5}M copper applied to the mucosal surface increased the potential from 6.4 ± 0.4 mV to 9.7 ± 0.5 mV (mean ± S.D. of six paired half bladders) and when treated with thyroxine, the bladders in normal saline showed no change, the value being 6.7 ± 0.5 mV one hour after hormonal addition. The bladders which had

10^{-5}M copper on the mucosal surface in addition to 10^{-6}M thyroxine on the serosal surface, showed a large increase in potential of approximately forty per cent, the increased value being 14.2±0.7 m Both sets of bladders showed the characteristic increase in short-circuit current when thyroxine at 10^{-6}M concentration was added to the serosal surface. Thus the reduction in anion permeability caused by the presence of copper increased the potential. After treatment with thyroxine in normal saline it is, therefore, evident that it is the anion movement which keeps the potential at steady values in the normal condition.

Some experiments were performed in an attempt to investigate any link between thyroxine action and magnesium ions, which has been reported to exist in mitochondrial work (Tapley and Cooper, 1956). The magnesium in the saline was replaced with a sodium salt, and the bladder washed continually. The flow of saline over the bladder was 5 cc./minute and was continued for thirty minutes. No difference could be detected either before or after thyroxine addition between bladders washed in normal saline, or those washed in magnesium deficient saline. Thus, it appears that the magnesium must be tightly bound within the cell, and without destroying the cell to expose the subcellular particles, work on the relationship of thyroxine with such metal ions in the whole cell appears unlikely to be profitable.

5. Tissue sodium and potassium content.

Attention was drawn to the Koefoed-Johnsen/Ussing model in the Introduction as a basis for explaining how bioelectric poten-

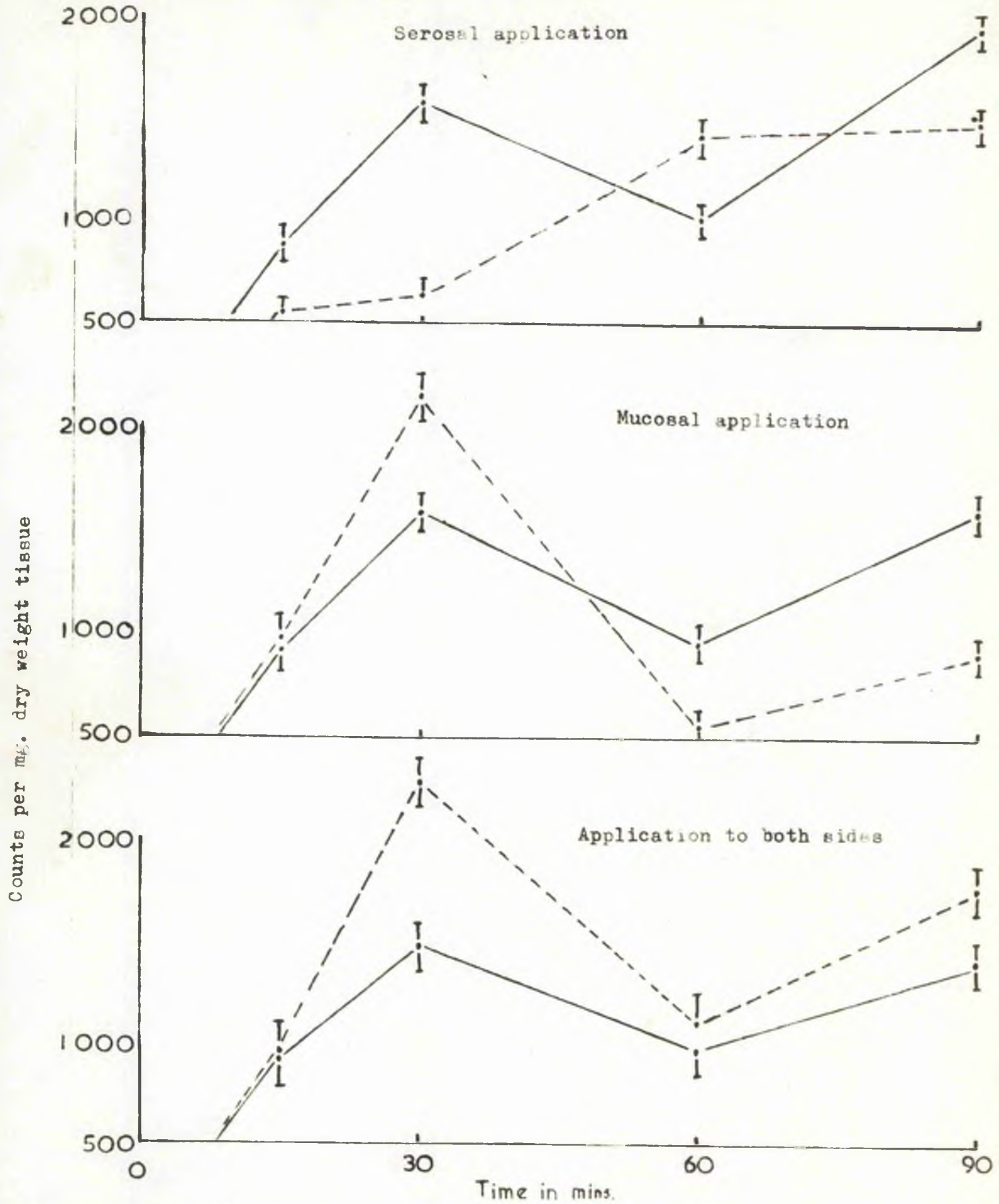
tials could develop. The present experiments were, therefore, performed to determine the effect of thyroxine on tissue sodium and potassium content and the relationship between the hormone and the permeability of the membrane.

Sodium content of the bladder.

The results show that thyroxine, when applied to the serosal surface of the toad bladder at 10^{-6}M concentration, lowers the tissue sodium content below that found in the control tissue, both at fifteen and thirty minutes. After sixty minutes, however, the tissue sodium content rises to that of the controls and then falls slightly after ninety minutes (Fig. 15). Fig. 15 also shows the effect of 10^{-6}M thyroxine on tissue sodium content when applied to the mucosal surface of the bladder. Within the first fifteen minutes it is impossible to detect any difference between the treated and control bladders. After thirty minutes, however, the tissue content has increased by a far greater extent in the treated than in the control tissue, thereafter the sodium content falls rapidly in the treated bladder below that of the control and then gradually rises to near that of the control tissues. The effect of thyroxine when applied to both sides of the membrane appears to be a combination of the two effects previously described (Fig. 15). Initially the sodium content appears to rise greatly and then falls at sixty minutes below the control tissue remaining lower than the control even after ninety minutes duration.

Potassium content of the bladder.

Thyroxine, applied to the serosal surface of the bladder at



g. 15. Effect of 10^{-6} M thyroxine on sodium (Na^{22}) content of the bladder, when applied to the different sides of the membrane. Treated ± S.D. - - - - , Control ± S.D. ——— .

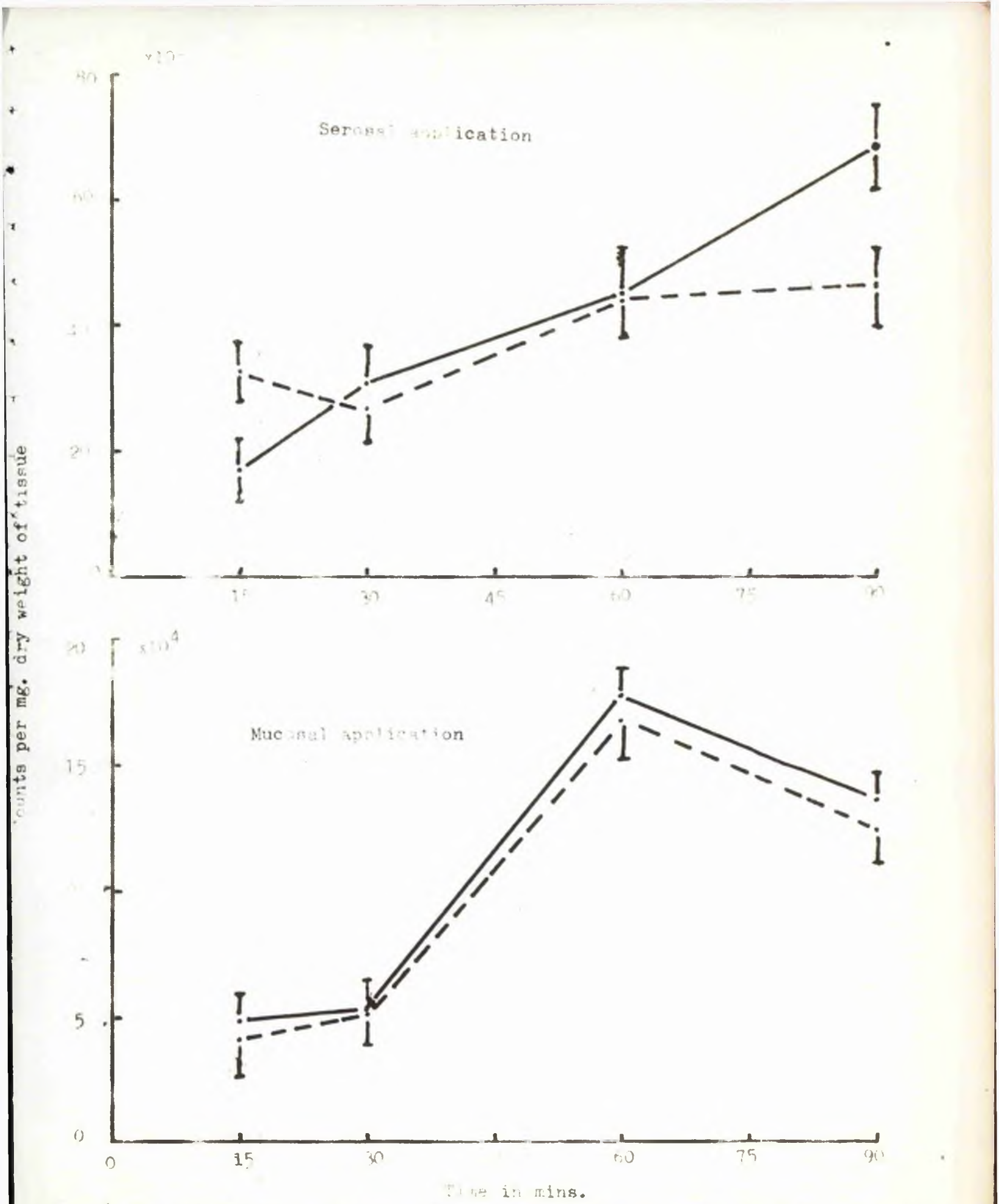


Fig. 16. Effect of thyroxine when added to the different sides of the toad bladder on the potassium (K^{42}) content of the tissue.
Treated ± S.D. - - -, Control ± S.D. ——.

10^{-6}M concentration, increases the potassium content above that seen in the control tissue within fifteen minutes after applicati (Fig. 16). After sixty minutes however, the content of the treated bladder becomes equal to that of the control, whilst at ninety minutes, the values show a difference, the treated value being lower. This is presumably due to the thyroxine being utilised from the incubation medium. Fig. 16 show the effect of a similar concentration of thyroxine on the potassium tissue content when applied to the mucosal surface. There is little difference between the values obtained until approximately sixty minutes when a slight decrease in the treated bladders is seen. The ninety minute values, show a similar difference between the treated bladders and the control values as that seen at sixty minutes.

In no case is any difference observed whether results are expressed as counts per mg. dry weight of tissue or as counts per mg. of tissue water (see Table 9). The mean water content of the bladders was 70%.

6. Analogues

None of the analogues used had any effect on sodium movement across the isolated toad bladder when used at 10^{-6}M concentration.

D. DISCUSSION

The net transport of sodium across both isolated bladder and skin of the toad is increased by addition of 10^{-6}M solutions of

Table 9. Comparison of counts/mg. dry wt. of tissue and counts/mgm. tissue water for both Na^{22} and K^{42} in the toad bladder, under control and treated conditions.

		Na^{22}				K^{42}			
		c/mg.dry wt.		c/mg. tissue water		c/mg.dry wt.		c/mg. tissue water	
		C	T	C	T	C	T	C	T
Thyroxine, serosal	15	830	510	270	174	17461	32758	4988	9099
	30	1530	630	530	221	31122	26996	8892	7296
	60	1000	1270	357	375	45199	46513	13293	12921
	90	1920	1300	681	391	68972	47560	19706	13588
Thyroxine, mucosal	15	900	970	310	330	49185	40587	13662	11599
	30	1590	2170	537	732	51040	50849	14582	14956
	60	950	550	320	181	168965	178099	48276	49371
	90	1600	870	521	288	136428	125377	38979	37333
Thyroxine, serosal and mucosal	15	920	960	307	330				
	30	1410	2290	471	703				
	60	1000	1180	348	357				
	90	1300	1650	450	571				

either thyroxine or triiodothyronine to the serosal surface. Definite stimulation of active sodium transport is also obtained at 10^{-8}M concentrations of these hormones. This observation is the first demonstration that the thyroid hormones, thyroxine and triiodothyronine, act on a sodium transport mechanism in membranes known to actively transport sodium. This, together with the previous work on water permeability, indicates that the thyroid hormones have a manifold effect on cellular permeability, both on active, energy-requiring systems and also on passive permeability to water in these tissues (see II).

The concentrations used were near-physiological, according to the data on mammalian plasma concentrations given by Pitt-Rivers and Tata (1959) where values of 10^{-7}M to 10^{-6}M are taken to be the levels of the hormone in the plasma. Definitive figures for thyroxine and triiodothyronine levels in Anuran plasma are lacking, although the presence of these hormones has been demonstrated in Amphibian thyroids (Shellabarger and Brown, 1959). The only results published concerning the Amphibia are those from the work of Genest and Adams (1957) where the secretion rate of male Triturus viridescens is given as 0.024 to 0.046 µgm of thyroxine per 100 gm. body weight. This, however, may be considered as a maximum level, for these studies were performed by injecting the minimal dose of thyroxine required to bring about moulting, at which time the needs for thyroid hormones are probably at their highest. The figures do show, however, that even at this high level they are comparable to those of the rat, differing only by relatively small factors.

The two hormones differ both in their time course of action and in the maximal response brought about by similar molar concentrations. Triiodothyronine causes a more rapid rise in short-circuit current, whilst thyroxine causes a greater maximal increase in sodium transport as well as occurring over a longer time interval. The situation as regards the relative speeds of action of the two hormones is similar to that found during in vivo mammalian work where triiodothyronine has a quicker action than thyroxine (Barker, 1956). When the 'total' activities are considered, however, a discrepancy may be seen for, whereas in mammals the two hormones have almost identical overall responses - as judged by the area under the curves (Tata, 1961, 1962), thyroxine causes a far greater response than triiodothyronine in this preparation.

Both the measured potential difference across the membranes and the transport potential calculated from isotopic studies remain virtually constant under both control and treated conditions. This occurs despite the demonstrated increase in sodium transport and a hypothesis of the action of thyroxine and triiodothyronine on membranes may be constructed.

Since directly measured active transport of sodium is increased when determined isotopically or electrically, then it is evident that the hormones increase the rate of turnover of the carrier system, whatever form this may take (see Ussing, 1955; Hokin and Hokin, 1961). The result of measurements of increased passive outflux of sodium, combined with the fact that the potential remains constant during the treated period, suggests that the permea-

bility to the flux of non-transported ions is altered in either direction through the membrane. Thus an increase in the movement of ions, particularly negative ions, would tend to maintain the potential virtually constant at the same level as that in the control membrane. The calculated fall in d.c. resistance of the membrane suggests that there are more ions per unit of tissue, for the presence of an increased number of ions means that transfer of electrical charge becomes easier. The results of chloride flux measurements indicate that there is indeed an increased movement of this ion species in either direction across the membrane under the influence of thyroxine, with a slight tendency for a greater increase in the direction of active sodium movement, that is, towards the serosal surface. The control flux rates of the phosphate ion are only slightly lower than those of the chloride ion. There is little difference between the measured potential when the membrane is bathed either with sulphate medium or with normal saline. Although no definitive values can be obtained from the literature, estimated values may be found which indicate that the sulphate and phosphate ions are of the same order of size and these are in turn a factor of twice the size of the chloride ion. As has been shown, the bladder treated with thyroxine increases slightly in potential when immersed in sulphate saline. Since, as the results show, the phosphate control flux differs little from the control chloride flux, the slight difference (assuming phosphate ion to be equal to sulphate) in ionic diameter and hence diffusion rate through the membrane, could account for this poten-

tial increase. When treated with thyroxine the chloride flux increases approximately two-fold whereas the phosphate permeability increases only by twenty per cent. Therefore, assuming the approximate values of ion diameter to be correct, it can be seen that the permeability to the sulphate ion is unlikely to rise greatly when treated with thyroxine. The explanation for the increased potential in the treated state, compared with the lack of increase wrought by substitution of sulphate ions for chloride ions in the control condition, is presumably to be sought in the membrane itself.

The sodium, being actively pumped, creates an electrical gradient across the membrane and this in turn causes anions to flow in the direction of this gradient. There is, however, a lag between the sodium and the anion movements and this results in the creation of a bioelectric potential. In the bladder bathed on each side with normal saline, the addition of thyroxine allows a freer passage of chloride ions through the membrane, thus equalising the effect of the increased sodium movement on potential. With sulphate medium present, however, although there is little difference between the normal fluxes of chloride and sulphate (assuming a direct comparison with phosphate ion), it appears that the sulphate ion reaches a point where the limit of its diffusion through the membrane is controlled by a permeability factor in the membrane rather than the gradient established as a result of the sodium pump. This factor presumably limits the amount of this larger ion whilst still allowing the smaller chloride ion to pass

through at a greater speed. Obviously, in non-stimulated conditions the sodium pump does not create a great enough driving force to make this factor of importance and it is only at higher rates of transport that the membrane factor exerts an influence.

This concept is supported by results from the membranes treated with copper. Ussin (1949 b) has shown that the presence of copper on the mucosal surface of frog skin decreases the anion permeability and presumably has the same effect in the toad bladder as judged by its effect on potential. These membranes showed an increase in potential compared with the control paired half bladders, which is due to the decrease in the anion shunt. Upon addition of 10^{-6}M thyroxine the normally treated membranes showed little or no increase in potential, whilst the copper treated membranes showed a forty-five percent increase in potential. Thus, by decreasing the anion permeability a rise in potential is found and a further increase occurs concurrently with a rise in short-circuit current under the influence of thyroxine.

It is thus established that thyroxine and triiodothyronine, in addition to increasing active sodium transport, increase the permeability of the membranes to anions, particularly the chloride ion. The increased sodium transport creates a greater driving force for the anions, and chloride ions are found to be able to move across the membrane more freely thus tending to nullify the increase in potential. Although differing only slightly from smaller anions in their permeability under normal conditions, the flux rate of the larger anions shows no great increase after hor-

mone treatment. This is due to some unknown factor in the membrane which limits their diffusion, because of their larger diameter compared with that of the chloride ion, once a certain rate has been achieved. The factor also, presumably, comes into effect on chloride ion movement, but at a much higher rate of sodium ion transport than can be reached with thyroxine stimulation. It may be significant that doses of vasopressin elicit changes in potential across the toad bladder (Bentley, 1960; Leaf, 1960 a) and it may be that at this very high rate of sodium transport a limit for the chloride permeability has been reached, resulting in an increase in potential.

A rather different picture is evident from the urea permeability fluxes. It has been suggested previously (Maffly, Hays, Lamdin and Leaf, 1960) that the urea moves across the toad bladder in aqueous channels. The evidence for this, is that many substances show low values for permeability, even when stimulated with vasopressin, but urea and water alone show large increases in their flux rates when thus treated. By comparison of the free diffusion coefficients for these two substances it was found that there was a close approximation between the ratio of the diffusion through the assumed channels in the bladder and the values in free solution. The evidence, therefore, points to the fact that urea moves through aqueous channels in the bladder. This being so, the increases in urea fluxes in each direction through the bladder, large as they are, support this concept and also show that 'pores' are being opened in the membrane allowing a freer

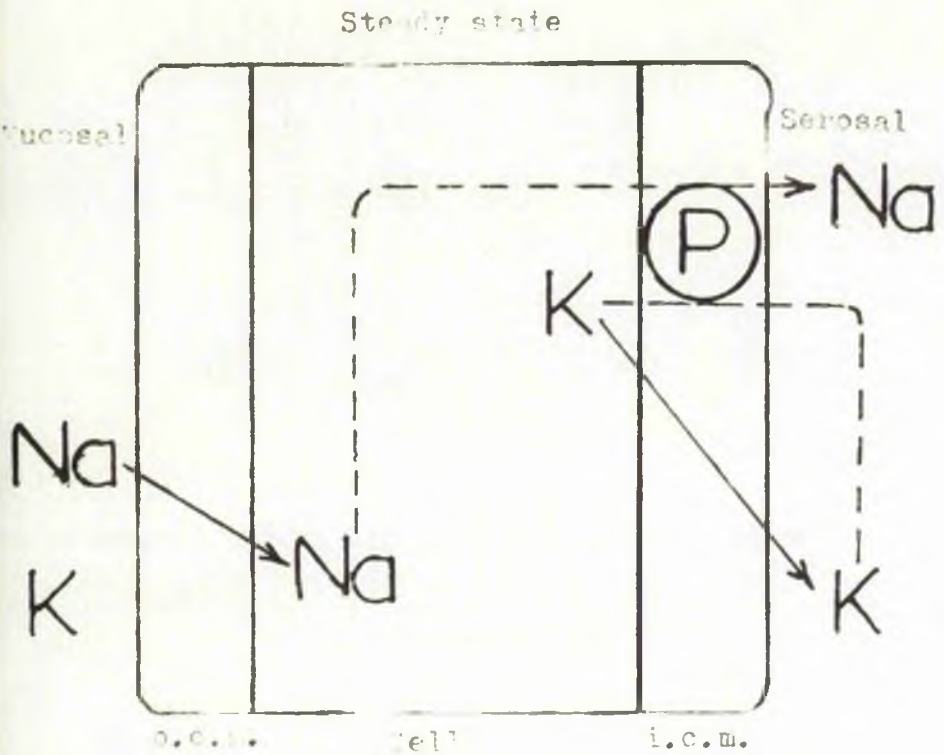
diffusion of water (Hays and Leaf, 1962 a). The mode of action of thyroxine at the membrane level seems, therefore, to be two-fold, firstly on the energy-requiring transport mechanism and secondly on the permeability of the membrane to other ions and water, the latter possibly brought about by alteration in pore size.

There are few detailed studies of hormone effects on ion transfer across isolated Amphibian membranes. Ussing and Zerahn (1951) showed that whale neurohypophyseal extract increases the sodium conductivity, whilst the transport potential remains constant. The measured potential difference and active sodium transport also increase in magnitude. A situation exists, therefore, which is similar to that found in the thyroxine treated membrane, except for the lack of increase in potential difference in the latter instance. Leaf (1961) has reported that the measured d.c. resistance of the toad bladder is not altered during vasopressin stimulation, although active sodium transport is increased. The effect, however, is variable and appears to be dependent upon the medium in which the bladder is bathed, indicating a complex effect of the hormone on passively moving ions. Crabbé (1961 b) found a slight decrease in measured resistance of the toad bladder when stimulated with aldosterone, together with a definite increase in active sodium transport. Other steroids have been investigated by Bishop, Mumback and Scheer (1961) who found a decrease in active sodium transport after adrenal tissue destruction, which occurred concurrently with an increase in d.c. resistance. It is of

interest that, in other circumstances, for instance, in Nitella and in the squid axon, it has also been found that physiological activation is accompanied by reduced electrical resistance (Cole and Curtis, 1938 a,b).

There are, however, numerous reports in the literature of effects of hormones on certain aspects of ionic movement across cell or tissue membranes. Although lacking in complete examination of hormonal effect, the majority of such studies indicate that many hormones influence various aspects of active transport systems whether of inorganic ions or of organic substances (see VI).

The effect of the thyroid hormones on sodium and potassium content of tissue can readily be explained on the basis of the Ussing model (see Fig. 17). Results from the experiments of thyroidal hormone effects on water permeability under isosmotic conditions indicated that the hormones were able to penetrate the cells at a faster rate from the serosal surface than from the mucosal surface. The sodium pump is located at the serosal surface of the cell (Leaf, 1960 a) and thus thyroxine when applied to this surface quickly enters the cell, resulting in an increase in transport of sodium, which leads to a depletion of tissue sodium content (cf. Fig. 17a). The higher rate of loss of sodium from the inside of the cell through the serosal surface creates a larger concentration gradient across the mucosal surface, and sodium is pulled into the cell. During this time the thyroxine has presumably penetrated into the cell and begins to act on the permeability of the mucosal surface thus further increasing the



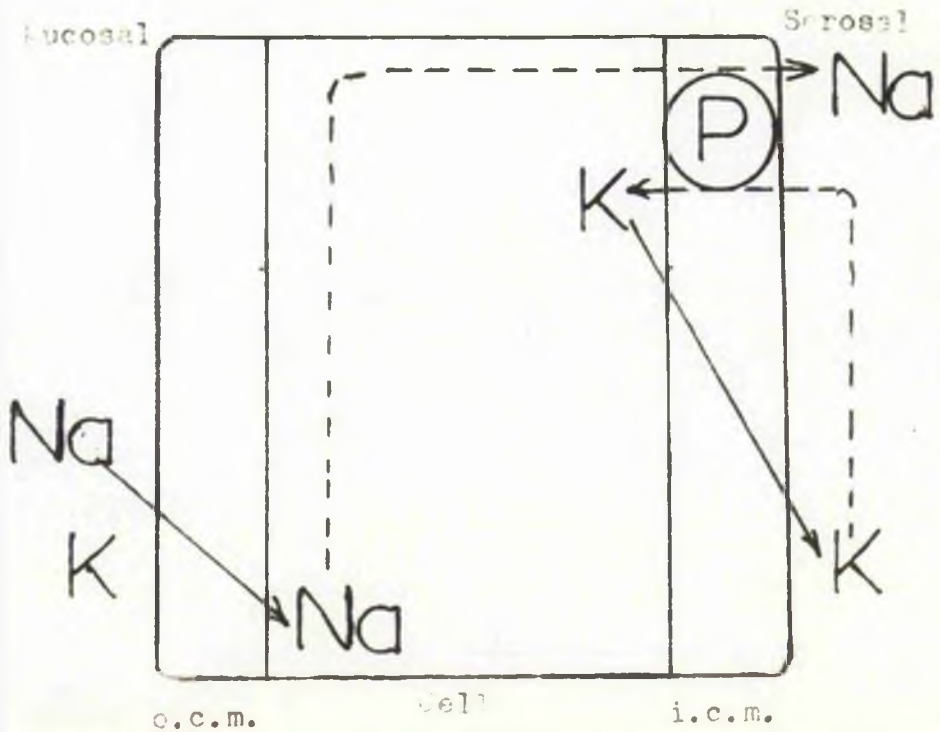
Key to symbols :-

o.c.m.	outer cell membrane
i.c.m.	inner cell membrane
Na	sodium
K	potassium
P	active sodium system

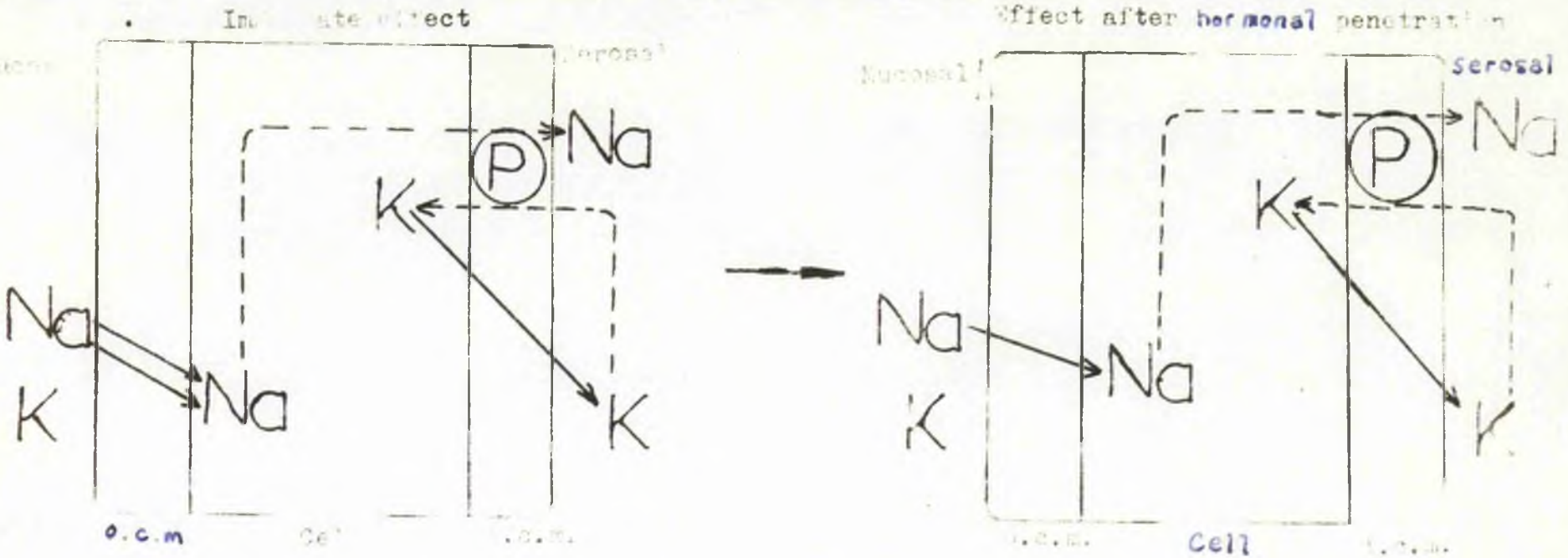
Height of the symbols from the base of the cell indicates either concentration (Na or K) or activity (P). In all diagrams it is assumed that the medium bathing the two sides of the membrane is similar in composition.

Fig. 17a. Theoretical action of thyroxine at the cellular level, based upon the Koefoed-Johnsen Ussing model of the developement of bioelectric potentials. The intracellular concentrations of ions are as found in the reported experiments, and the height of the sodium pump is as seen after the addition of the hormone to the different sides of the membrane, and is based upon the measured short-circuit current.

Thyroxine on the serosal surface.



Thyroxine on mucosal surface



Thyroxine on both sides

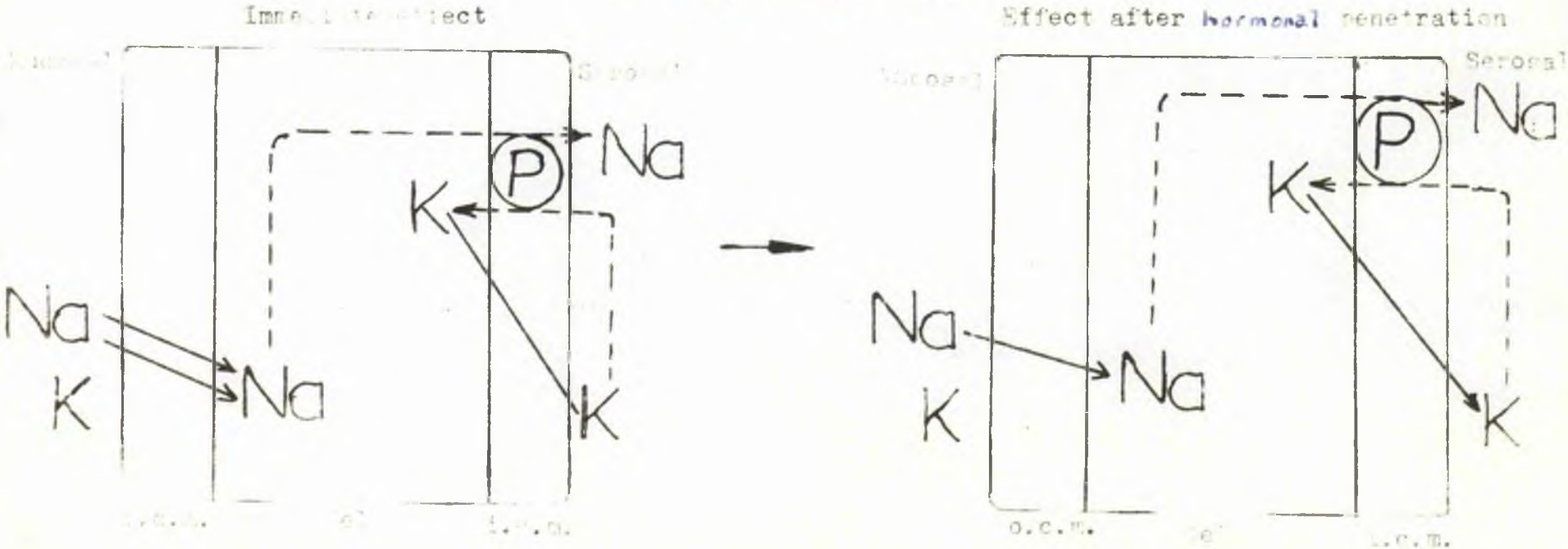


Fig. 17b. For explanation see Fig. 17a.

permeability of the cell. Although the turnover of sodium is higher than that of the control tissue, the content of labelled sodium is no higher for it is being moved through at a faster rate.

When thyroxine is applied to the mucosal surface, the immediate effect is on the passive permeability to sodium across this membrane (cf. Fig. 17 b) and the cell receives a larger amount of sodium. This increased availability of sodium itself, results in a slight increase in the activity of the sodium pump. The hormone, having penetrated into the cell, then acts slightly on the sodium pump to cause a decrease in the tissue content of sodium. These studies are, however, complicated by the fact that the effect of thyroxine is completed within one and a half hours, thus towards the end of the experimental period, a readjustment is taking place. They do, however, offer an analysis to be made on the ionic constituents of the cell.

The potassium content has similar relationships, as is predicted by the Ussing model for forced exchange of sodium and potassium across the serosal surface membrane. For when thyroxine is applied to the serosal surface the potassium content rises initially, showing a relationship with the measured increase in short-circuit current and potassium exchange, and later falls when the hormone effect decreases. When applied to the mucosal surface thyroxine causes a slight increase in potassium content after sixty minutes, due to its indirect effect on the sodium pump. There is little difference, however, between the treated and control tissues, which is again as predicted by the Ussing model as

a basis for the explanation of the experimental results. These results also support the concept of the model, for the sodium-potassium link is shown to exist by these isotopic measurements.

With a thyroxine-triiodothyronine mixture a biphasic curve is obtained which shows a significant shift of the thyroxine part of the curve towards the point of addition of the hormones. Thus a further indication is obtained of their ability to act together, although differing in their time course of action. The triiodothyronine presumably acts by increasing the penetration of thyroxine into the cell or onto cell surface receptor sites.

The work on the skin of the toad is interesting in relation to work on moulting and associated effects. Kaltenbach (1949) and Clark and Kaltenbach (1961) working on urodeles showed that thyroxine acts directly on the skin of the newt and causes moulting. In the Anura, Jørgensen and Larsen (1961) have suggested that in the Bufo species thyroxine may be acting synergistically with other hormones, particularly those of the adrenal complex. Koefoed-Johnsen and Ussing, (1949) made a few observations on the effect of injected thyroxine on salt uptake of the axolotl and found that thyroxine caused an increase which was quantitatively less than that brought about by ACTH. Furthermore, Jørgensen (1949) showed that during moulting the sodium influx was increased. It may be reasonable to suggest from the present work on isolated skin that thyroxine, known to cause moulting in certain Amphibia, may also be responsible for the increased influx of sodium observed at these times.

These experiments show that the thyroid hormones, thyroxine and triiodothyronine, influence an actively transporting system in membranes, namely that of sodium, which is dependent upon metabolic energy derived from the cell, therefore, these hormones must be increasing the activity of the energy sources which are directed towards the working of the pump. Maffly and Edelman, (1963) have suggested from their work on toad bladder that the energy source of the sodium pump is only in part connected to the general metabolism of the cell, and may consist of some vectorial arrangement of enzymes in the membrane which is responsible for the translocation and the observed anisotropic distribution of the ion such as that proposed by Mitchell (1961, 1962). These observations bring the thyroid hormones into a similar category to other hormones which have been shown to effect the permeability of isolated membranes, thereby presenting more evidence that hormones of diverse structure and size act on the same basic cellular processes. These increases in permeability suggest that structual alterations are taking place in the membrane and it may well be that this is one of the fundamental effects of thyroxine at the cellular level. Cellular permeability to amino acids is also increased by thyroxine, as has been shown by Tata, Ernster and Lindberg (1962) and these authors conclude that control of the basal metabolic rate by thyroxine appears to be a consequence of a general action at the cellular level. This explanation also provides an opportunity to reconcile many of the extremely varied actions of these hormones at the tissue level (see Pitt-Rivers and

Tata, 1959). These results from mammalian work and the present results indicate that the thyroid hormones exert a control of the close relationship between cellular structure and function.

SECTION IV.

Effects of thyroidal hormones on the oxygen consumption of isolate toad tissues.

A. INTRODUCTION.

Since the demonstration in 1895 by Magnus-Levy that the feeding of thyroid extract increased the oxygen consumption of human patients, much work has been performed to further investigate this problem of the metabolic effects of thyroidal hormones, both *in vivo* and *in vitro*. It is undisputed that an effect of the thyroid hormones exists on oxygen uptake in mammals treated *in vivo* but in the lower vertebrates the picture is by no means clear. Many attempts have been made to determine whether the thyroid hormones affect respiratory metabolism and there are several contradictory reports in the literature concerning the thyroid effect on fish and amphibian metabolism (Pickford and Atz, 1957).

Perhaps one of the most significant reports is that by Muller (1953) working with goldfish. He obtained highly significant increases in oxygen uptake after single injections of thyroxine, which reached a peak after five or six hours and continued injections raised the metabolic rate far above that of the saline-injected controls. Results similar to these were obtained by injection of thyrotrophin which indicated once more that thyroxine, or stimulation of release of thyroxine, caused a rise in respiratory metabolism. Similarly, a two hundred and twenty percent increase in the respiration rate of the goldfish was obtained by Chavin and Rossmoore (1956) after injection of thyrotrophin, although no effect upon oxygen uptake could be discerned after

thyroxine injection. A similar stimulation was obtained by Smith and Matthews (1948) using an extract of fish thyroid instead of the more usual technique of using mammalian or synthetic thyroxine. In contrast, however, negative results have been obtained by many authors on other teleost fishes (Drexler and Issekutz, 1935; Root and Etkin, 1937; Smith and Everett, 1943).

Studies of the effects of thyroidectomy and thyroid inhibition in fish have also given conflicting results. Neither surgical removal nor radiothyroidectomy of the thyroids of parrot fish (Matty, 1957) and trout (Fromm and Reineke, 1956) respectively, affected their respiration. The work using antithyroid materials must, however, be treated with caution for the exact effect of these substances on tissues other than the thyroid is, as yet, unknown. The use of such goitrogens has been critisized by Matty (1960), who pointed out that there are other deleterious effects of these compounds to be considered within the animal. Hickman (1959) found, by direct observation, that there was a correlation between the thyroid activity and the metabolic rate of the flounder. This appears to be the first observation of a metabolic effect of thyroid secretions not using administered materials and is of great value for this reason.

Little work has been performed on the elasmobranchs, but Matty (1954) did not observe any effect on the oxygen consumption of Scyllium even forty-two days after surgical removal of the discrete thyroid gland found in this group. The thyroid extract from these fish, however, caused an increase in the respiratory metabol-

ism or rats, which indicates that the thyroid secretions do hav metabolic effect at least in other animals. Pritchard and Gor (1960), in contrast, found that injections of triiodothyroacetic acid elevated the oxygen consumption of late embryos of the spin dogfish. The increase was of the order of twenty percent above the oxygen uptake of the control animals, but fell after four to five days despite continued injections.

In the Amphibia, Warren (1940), feeding frogs twice weekly thyroxine noted an increase in metabolism as measured by weight crease as well as oxygen consumption. This metabolic effect, de termined in this case by a directly measured increase in oxygen consumption, has been confirmed recently by Donoso and Trivelloni (1958) after injection of toads with either triiodothyronine or thyroxine. After thyroidectomy of newts, Taylor (1936) found a gradual decline in oxygen consumption similar to that found in mammals after similar surgical treatment. In direct contrast to the work described above, Galton and Ingbar (1962 a), also working on frogs, failed to obtain an increase in respiration rate with either the parent hormones or several analogues. Henschel and Steuber (1931) also found no increase in respiration of winter frogs after treatment with thyroxine, although they also investigated the effect on summer frogs, working with groups of animals to eliminate individual variance. In this latter case, however, they obtained an increase in respiratory quotient and the calorie production per gram per day was also slightly increased.

The divergent results obtained from <u>in vitro</u> work in all

groups of animals are confusing, for there are reports of stimulation of respiration, and others that there is no effect at all. Wiswell, Zierler, Fasano and Asper (1954), who investigated the effect of addition of both triiodothyronine and thyroxine to rat liver and diaphram, found no increase in respiration rate, a result which was also obtained by Wiswell and Asper (1958) when investigating the respiratory effect of TRIAC and TETRAC. Goh and Dallam (1957) found no metabolic effect of thyroxine on the isolated rat heart and Radsma, Golterman and Birkenhager (1954) obtained a similar result using rat liver homogenate. Weiss (1956) explored the effects of both triiodothyronine and thyroxine on oxygen consumption of skeletal muscle from turtle, guinea-pig, dog and man. Despite the diversity of tissue which he used, no increase in metabolism could be detected. Hoover and Turner (1954) investigated the effect of addition of thyroxine and triiodothyronine on rat mammary gland, but found no stimulation of oxygen uptake. The report of Cereijo-Santalo, DiNella, Park, Park and Pitt-Rivers (1958) indicate that decreases of varying degree in oxygen uptake of Erhlich ascites tumour cells are obtained with a wide variety of analogues of the thyroid hormones. In contrast to these negative results, stimulation of oxygen consumption <u>in vitro</u> by thyroxine has been reported by earlier authors in the period 1922-1936. The reports of Scott (1935) on alligator red blood cells; Davies, DaCosta and Hastings (1934) on rat heart; Verebely (1932) on rat skeletal muscle, and both Reinwein and Singer (1928) and Mansfeld (1935) all show that thyroxine has an

immediate effect on respiration. After this initial period, where the above authors obtained the increases in respiration with very small doses of thyroxine, much work was performed which almost always gave negative results. This negative work has been offset by reports from Gross and Pitt-Rivers (1953) on rat kidney; Yoshihiro (1956) on dog adrenal cortex; Grief and Moroney (1959) on rat kidney slices and Jacob (1962) using rat liver slices. Weinstein and Lein (1957) observed that a certain time after the addition of thyroxine to the medium the respiration was augmented, but at highe thyroxine concentration a depression of respiration was seen.

Certain analogues of the thyroid hormones have also been shown to stimulate oxygen uptake in vitro. Alexander and Bisset (1958) and Bisset and Murray (1962), for example, obtained an increase in oxygen consumption of leucocytes from myxoedematous patients when treated with TRIAC, whilst van Zyle and Engelbrecht (1959) also using TRIAC found an increased uptake of oxygen by rat kidney cortex. The interesting report of Hamolsky, Michel, Garnicerno and Roche (1963) indicates that thyroxine and triiodothyronine produce a slower and lesser, but nevertheless significant, increase than TRIAC in stimulating horse leucocyte oxygen uptake. Shortly after the isolation of TRIAC and TETRAC, Thibault and Pitt-Rivers (1955) demonstrated that both these analogues had immediate effects on metabolism of rat kidney cortex in vitro. Both Barker and Lewis (1956) and Wiswell and Asper (1958), however, failed to confirm these findings in rat tissues.

Reports of in vitro work on Amphibian tissues are very scarce,

but although Wiess (1956) reported that neither thyroxine nor triiodothyronine caused an increase in oxygen uptake of frog skeletal muscle, both Ahlgren (1925) and Haarman (1936) found a stimulation of tissue respiration after immersion of frog skeletal muscle in thyroxine solution. Haarman's work is of particular interest because of the extremely low doses with which he worked, for he found the optimum concentration of thyroxine was 10-15 gm./ml. (8×10^{-16}M). These three isolated studies appear to be the only ones in existence concerning the effects of thyroid hormones upon metabolism when applied to Amphibian tissue in vitro, except that of Thornburn and Matty (1963), working in this laboratory, who have confirmed the findings reported in this section.

Because of the well-known calorigenic effects of the hormones in mammals, studies have been made on the intracellular enzyme systems which exert a control on tissue respiration and have yielded much contrasting information. Such studies have been carried out for the past decade, but the inconsistencies are so fundamental that doubt has been expressed whether a direct hormone enzyme interaction exists. Further, it has recently been suggested that many of these changes in enzyme activity are, in fact, secondary to an effect on intracellular membranes or binding with metallic ions (see Tapley and Hatfield, 1962). In the carefully controlled experiments of Fairhurst, Roberts and Smith (1959), who worked on different cell fractions of the rat liver, a stimulation of respiration of homogeneous mitochondrial preparations was found, without concurrent effects on the exchange of fixation

rate of inorganic phosphorus. The results of this work indicate a direct control of thyroxine on mitochondrial respiration. The work of Tata (1962) has, in addition, shown that the thyroid hormones affect oxidation processes occurring in the mitochondria of rat liver and muscle, as well as microsomal enzymes which are concerned with protein synthesis.

A limited amount of work has been performed on the respiratio of the isolated toad bladder and it has been shown (Leaf, Page and Anderson, 1959) that of all the toad tissues, only the oxygen uptake rate of the intestine surpasses that of the bladder. The respiration rate of this latter tissue is twice that of liver and skeletal muscle and three times that of heart, skin and stomach. It is worth noting that some of the negative results noted in the past in studies on thyroid hormone effects, may arise from the fact that investigators have used Amphibian tissue of low normal metabolism.

The toad bladder has already been shown to actively transport sodium (Leaf, 1955; Leaf, Anderson and Page, 1958; III,C,3) and also to respire aerobically. Upon reduction of the sodium content of the medium bathing the bladder to insignificant levels, the oxygen consumption also falls to a lower basal rate (Leaf, Page and Anderson, 1959). With a bladder in normal saline, a stimulation of oxygen consumption is seen after the addition of vasopressin. Under conditions where there is no sodium present in the medium, however, vasopressin administration causes no increase in the oxygen uptake (Leaf, Page and Anderson, 1959; Leaf

and Dempsey, 1960). It appears, therefore, that the action of vasopressin on metabolism is a result of its effect on the energy-dependent sodium transport system across the bladder.

Zerahn (1956, 1958), using frog skin, was able to show that there exists a close relationship in this tissue between the oxygen molecules consumed and the sodium ions transported. He demonstrated this by first bathing the tissue on both sides with sodium-free saline and thereafter measuring the 'basal' respiration rate. After the addition of sodium, the respiration rate was stimulated as a result of the activation of the energy-dependent processes underlying the sodium pump. By measurement of the increase in sodium transport and the increment in oxygen uptake caused by the presence of sodium, the close relationship between oxygen uptake and sodium transport was obtained. Leaf and Renshaw (1957) were able to verify this work using a different technique. They measured the basal respiration rate in sodium-containing saline before and after addition of vasopressin. The measured increases in oxygen uptake and sodium transport show the oxygen consumption and ion transport to be proportionally related. It was readily seen that the relationship between oxygen uptake and ion transport holds during these periods of increased metabolism. The increased oxygen requirements under both these conditions, that is, when sodium is applied to a membrane bathed in a non-sodium-containing medium and when sodium transport is stimulated with vasopressin, is a reflection of the metabolic requirements of the sodium pump.

Since the urinary bladders of Bufo marinus and Bufo bufo have also been shown to transport sodium actively and in addition such a close relationship between oxygen uptake and ion movement holds, at least, in Bufo marinus bladder (Leaf, Page and Anderson, 1959), the effects of thyroxine have been investigated on bladder respiration in a sodium and a sodium-free medium. It has been shown by Maffly and Edelman (1963) that only a part of the general cellular metabolism is directed towards the sodium pump and, in order to investigate the effects of thyroxine on that part of the metabolism not directed towards sodium transport, experiments have been performed using a sodium-free saline. Thus correlation has been sought between the proven increase in active sodium transport across the bladder (III,C) and the metabolic effects of the thyroid hormones, which are known to differ from other hormones in their effects.

In the vertebrates, thyroxine and triiodothyronine are known to directly alter general cellular metabolism. Insulin is also known to increase the respiration rate of certain tissues, but this is a reflection of its effect on glucose transport across cell membranes and the general utilisation of glucose. Hormones which affect transport of substances across tissues, such as vasopressin and insulin, have also been shown to affect the metabolism of the particular tissue. Such increases are reflections of the stimulation of the kinetics of the transport of a particular substance, which is dependent upon glycolytic energy and thus, in periods of increased energy requirements, has a greater need for

oxygen. Although the recent studies of Goodfriend and Kirkpatrick (1963) have demonstrated that oxytocin and vasopressin stimulate the metabolism of the toad bladder independently of any effect on sodium transport mechanism, the results show that only a small fraction of the total stimulation is independent of the requirements of the sodium pump.

The corticosteroids are also known to increase tissue metabolism but this effect is a catabolic one rather than that of anabolism seen as a result of the presence of thyroid hormones in the vertebrate. The action of the latter substances in physiological quantities is more on growth and development, changing to a catabolic process only under stress. Tata, Ernster, Lindberg, Arrhenius, Pedersen and Hedman (1963) have ably demonstrated that the use of physiological doses of thyroid hormones results in a 45-75% increase in metabolic rate, as well as a stimulation of body growth. This stands in contrast to a great deal of work in which higher doses of thyroxine have been used and a fall in body weight occurs concurrently with a rise in metabolic rate. The physiological effect, therefore, appears to be anabolic, resembling that seen in Amphibian metamorphosis which is again associated with increased thyroid activity (Lynn and Wachowski, 1951).

B. MATERIALS AND METHODS

1. Animals

Mature toads (Bufo bufo) of both sexes were used in these investigations, obtained either from a commercial source (L. Haigh and Son, Surrey) or collected locally. Upon arrival they were placed in a constant temperature room at 12°C. The animals obtained commercially were not fed during the short period for which they were stored before utilisation, for they were not kept for longer than fourteen days after arrival. The animals which were collected locally were also kept in the constant temperature room, but every two weeks they were force-fed with an egg-liver homogenate and, in addition, fed twice weekly on flies or maggots. Animals were not used during the two-day period after arrival.

2. Saline solution

The saline solution used had the following composition:- NcCl 6.5 g./l., KCl 0.2 g./l., $CaCl_2$ 0.2 g./l., $MgSO_4$ 0.1 g./l., $NaHCO_3$ 1.5 g./l., Na_2HPO_4 0.1 g./l. and glucose 0.75 g./l.

The pH was normally 7.8 and was always adjusted to this value where necessary with the appropriate KOH or HCl solution. The osmolarity was 260 milliosmoles. Fresh saline was made up every three days during the experiments, and more often if required, and stored in stoppered flasks at room temperature.

3. Apparatus

Two techniques were used for the measurement of the oxygen consumptions of the membranes used. Initially the oxygen uptake was determined using a dropping mercury cathode (Cambridge Instrument Co.). This technique relies on the fact that when a voltage is applied between the two poles of a circuit with a pool of mer-

cury as the anode and a constantly forming and reforming mercury cathode, oxygen is reduced at the cathode and electrons are caused to flow in the circuit. The current measured is proportional to the oxygen content of the solution concerned. The anodic pool of mercury is made large enough to be non-polarisable whilst the cathode consists of drops of mercury which emerge under a constant head of pressure from a piece of fine capillary tubing. The applied voltage to be used in such determinations is readily found by analysing the voltage-current relationship of a standard solution. It is initially found that when electrolysis of the solution begins the current rises rapidly as the applied E.M.F. is increased. As the E.M.F. is further increased, the rate of increase falls and a flattening of the ensueing curve is seen, this being a point where the limiting process is the rate of diffusion of the substance into the cathode layer and as such is independent of the applied E.M.F. The current therefore, remains constant until a further reduction process takes place at higher voltages. The rate of diffusion depends directly upon the concentration of the substance being determined, therefore, the diffusion current is a direct measure of this concentration. The wave height for oxygen is roughly 0.6 to 0.7 volts, this value being approximately midway along the flat portion of the curve and this voltage was applied between the two mercury poles. It is convenient that the wave height for oxygen masks those for other substances and therefore makes analysis easier. Using the Cambridge instrument a constant head of mercury was easily obtainable, using the adjustable

reservoir of mercury, and thus a contant rate of formation of mercury drops was obtained.

When using this technique it is essential to prevent the mercury coming into contact with the tissue, for mercury has a deleterious effect on tissues and would most certainly kill the tissue when in contact for a long period of time, as would be required in this type of experiment. In order to prevent this contact occurring, a glass vessel was made which had a narrow hole at its base. Underneath this was a cup, sealed onto the glass to form a separate holder for the anodic pool of mercury. It was intended that the cathodic drops of mercury were to fall through the solution, in which the tissue was suspended on a glass hook, and then to fall through the hole in the glass holder into the anodic pool. This procedure, however, proved to be unsuccessful as the mercury did not always enter the anode, thus leaving the mercury in the solution, resulting in the expected effects on the tissue. Sealing the vessel also proved difficult, for it is essential that no gaseous exchange should take place with the atmosphere, but as mercury was continually falling into the fluid, excess fluid must be allowed to flow out. This aperture allowed gaseous exchange to take place.

A different approach was therefore tried and this proved successful. Small 1.5 ml. holders were made which fitted into a water bath supplied with the polarograph. These had a small piece of platinum wire sealed into the base and were thus connected to the anode of the circuit. The soldered connection was

sealed with 'Araldite' thus making the joint water-tight, the platinum-glass joint was also covered with the resin to render electrical leakages impossible. A pool of mercury, 0.4 ml., was placed in the bottom of this holder and the whole suspended in the water bath. The cathode has already been described.

The relationship between current measured and oxygen content of the solution was determined using saline solutions which had been bubbled with nitrogen (previously bubbled through pyrogallol to remove all traces of oxygen) and various nitrogen/oxygen mixtures up to air concentration of oxygen. Finally air was bubbled through the saline and the resulting concentration was analysed. The settings of sensitivity were such that the oxygen concentration in saline at 25°C., which had been bubbled with air, gave 80% of full scale deflection on the galvanometer. A linear relationship was found from a graph plotted of galvanometer reading against oxygen content of the solution (as determined by a Winkler analysis from samples of the solution used). This was checked periodically using different solutions, but little variance was found as long as the temperature was within 1°C. of that at which the original determinations were carried out, namely 25°C.

The other technique utilised was that of vibrating platinum electrodes, as used by Leaf and Renshaw (1957). These were incorporated into the apparatus I and II, described in III,B,3. The platinum electrodes were made from 'Pyrex' glass tubing, 3" length, with a piece of platinum wire sealed into the end. The sealed end was then cut flat using a tungsten-carbide tipped

dental bur and polished with a fine diamond disc. Polishing was then performed using fine carborundum particles suspended in distilled water, and examined under a microscope. If the electrode face was smooth and no irregularities could be seen, the electrode was accepted for use; if, however, the face showed any signs of 'pitting' it was subjected to more polishing until such irregularities had been removed. Originally these were ground and polished by hand, primary grinding being performed with emery paper, progressing through fine carborundum particles suspended in water until the final polishing, which was achieved using a soft cloth which had been immersed in a liquid polish ('Brasso'). The glass tube was then filled with mercury from a syringe thereby making the elimination of all air bubbles an easy operation. A small length of stainless steel wire was silver-soldered to ordinary wire, the steel wire was then pushed into the mercury and sealed in this position with 'Araldite'. The electrodes were periodically checked for cleanliness and 'pitting' and were polished, when in daily use, once each week.

The two platinum electrodes, one in each chamber, were connected independently to the negative pole of two polarograph circuits and the circuits were completed through agar-saline bridges leading from the chambers to silver-silver chloride electrodes. In apparatus II, these electrodes and bridges were built into the blocks of perspex (see Fig. 10). A voltage of 0.65 volts was applied across each platinum electrode and its corresponding silver-silver chloride electrode. At such a voltage across a

rapidly moving noble-metal microelectrode only molecular oxygen in the solution will remove electrons from the platinum cathode and its use has been validated by several workers (Longmuir, 1954; Chance and Williams, 1955; Leaf and Renshaw, 1957; Connelly, 1957). Under these conditions the current flowing in the circuit is directly proportional to the concentration of oxygen in the medium.

The microelectrode was forced through a tight polythene collar into the chambers such that about 0.5 cm. of the glass tubing projected into the chamber. The portion of the electrode above the chamber was vibrated laterally at 50 cycles per second so that the platinum tip inside the chamber was displaced two to four mm. To ensure a steady rate and amplitude of vibration during the measurements a modified Mickle shaker (H. Mickle, Gomshall, Surrey) was used. Perspex holders were made for the electrodes and were connected to one arm of the vibrator (see Fig. 18). Lindsey (1952), who investigated the basic characteristics of such microelectrodes, has shown that the diffusion current is proportional to the frequency of vibration up to about forty cycles per second, whilst from forty to one hundred and twenty cycles per second the diffusion current is independent of the frequency. In addition, he also demonstrated that the diffusion current is proportional to the amplitude when a large amplitude is used in conjunction with a low frequency. As the tip speed increases, the diffusion layer at the cathode decreases until it reaches a limiting value. The frequency of fifty cycles per

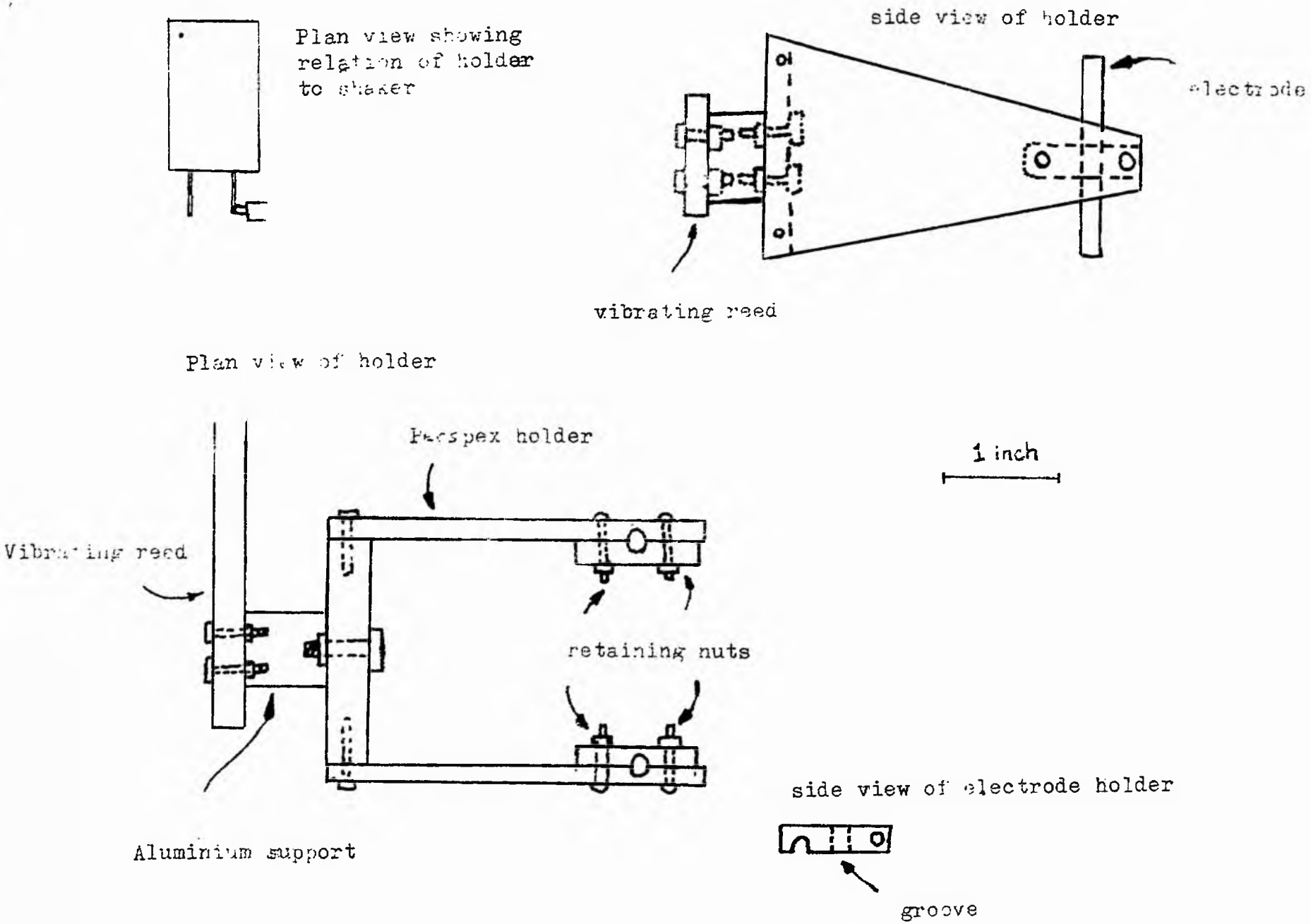


Fig. 18. Diagram of the adaptations made to a Mickle shaker for holding the vibrating electrodes during determinations of oxygen consumption.

second coupled to the amplitude of two to four mm. gives an average tip velocity of 44.4 or 88.8 cm./second, which is well above the minimum values laid down by Lindsey's work.

To determine the relationship between oxygen concentration and the diffusion current the electrodes were inserted into the saline-filled chambers with no tissue present. The air or nitrogen/oxygen mixture was then bubbled through the saline via the circulation system and the particular mixture was allowed to equilibrate with the saline for thirty minutes. In apparatus I the inlets and outlets of the chamber were quickly clamped off with artery clamps and the galvanometer deflection recorded over a period of thirty minutes. In apparatus II the greased perspex plugs were inserted into the appropriate holes and again the reading observed for thirty minutes. The stability of the recorded value served as a check against air leaks in the system. Since oxygen concentrations inexcess of those in air were not to be used, the galvanometer was adjusted to give a deflection of eighty percent full scale at this value at 25°C. The oxygen content of each solution used during the calibration was determined by a Winkler analysis. The zero value for the electrodes was determined by bubbling cylinder nitrogen through a pyrogallol solution to remove all traces of oxygen. This was bubbled into the solution, and allowed to do so for thirty minutes, to wash out all traces of oxygen from the solution in the chambers.

4. General procedure

The bladder or ventral skin was removed from the animal by

the methods already described, II, B and III, B and placed in a petri dish containing saline at 25°C.

Using the dropping mercury electrode technique only bladder consumptions were determined. The bladders were opened to form flat sheets exposing mucosal and serosal surfaces and washed for forty-five minutes in aerated normal saline, after which they were introduced into a 10.0 ml. all-glass syringe containing saline equilibrated with air at 25°C. The syringe was first filled with this saline and the bladder dropped in, care being taken that there were no air bubbles in the bladder, the plunger was then placed into the barrel and the volume adjusted to 10.0 ml. The syringe was gently agitated in a 25°C. water bath with the needle sealed by a small piece of rubber. Aliquots of 1 ml. were taken every five minutes for polarographic analysis, the volume required being ejected from the syringe into the 1.5 ml. holders described. Analysis was performed immediately upon removal of the sample from the syringe. It was assumed that any gaseous exchange between the sample and the air was slow enough not to affect the readings. The galvanometer deflection varies with the size of the mercury drop at the end of the capillary tube and is, therefore, constantly swinging between two points. When the galvanometer deflection returned to one reading for three consecutive swings it was taken that this value was the true one and thus recorded. The number of samples which could be taken was nine, thus limiting the total period of time over which samples could be taken to forty-five minutes. Determinations were therefore carried out on bladders

which had been immersed in saline for forty-five minutes after the washing period, by the same procedure as described above. Similar studies were also made on bladders treated with thyroxine at 10^{-6}M concentration, the bladder being added to a syringe containing normal saline plus thyroxine at 25°C.

For the measurement of oxygen consumption using the vibrating electrodes, the half bladder or piece of skin was placed so as to divide the chamber into two halves and the two halves of the chamber were clamped tightly together. The electrodes were set up with their holders and after filling the chamber with saline, the electrodes were vibrated. It was found that a period of fifteen minutes was required before the electrodes became stable and gave a constant reading. The membrane was allowed to equilibrate for forty-five minutes and then the circulation system was shut off. In apparatus I this was achieved by clamping the inlets and outlets of the chambers with artery clamps, the drop in oxygen content of the saline being measured during the control period. The saline was then allowed to regain the oxygen concentration of the air at that particular temperature and the hormone was added, either as a concentrated solution or as a solution of the required concentration already made up. The chambers were clamped off at this time also and the drop in oxygen content of the saline was again measured during this period. The same procedure was used when using apparatus II, but in this case the circulation system was removed and the openings in the block quickly sealed with greased perspex plugs.

With each method of determining the oxygen consumption, a standard curve was prepared using saline plus thyroxine and this showed no deviations from the curve obtained with normal saline. The temperature of the saline during the experiments using either apparatus I or II was 20 to 25°C. Whole bladders or pieces of bladder or skin (depending on the system used) were removed from the apparatus and, in the case of the pieces of bladder or skin in either apparatus I or II, trimmed to the exact internal area exposed to the saline, and dried to constant weight at 105°C. The results obtained using the microelectrode method of oxygen consumption are not significantly different from those using the dropping mercury cathode. The lowest reading of oxygen in the saline at the end of an experimental run was 23.5%. Constant oxygen uptake has been shown for isolated frog muscle when the partial pressure of oxygen is reduced from 760 mm. to 0.5 to 2.0 mm. mercury (Hill, 1948). It is unlikely, therefore, that the fall in oxygen content of the saline would alter the consumption of the tissue. Using the electrodes it was found that readings taken every 2.5 minutes enabled the hormonal effect to be followed very accurately.

5. Circuit

The circuit for the polarograph used in conjunction with the vibrating electrodes is shown in Fig. 19. The voltage source, a six volt battery, is connected to two potentiometers R_1 and R_2 of 1,000 and 10 ohms resistance respectively and serve as a coarse and fine adjustment of the applied E.M.F. The voltmeter, V, enabled the applied E.M.F. to be read to the nearest twentieth of a volt.

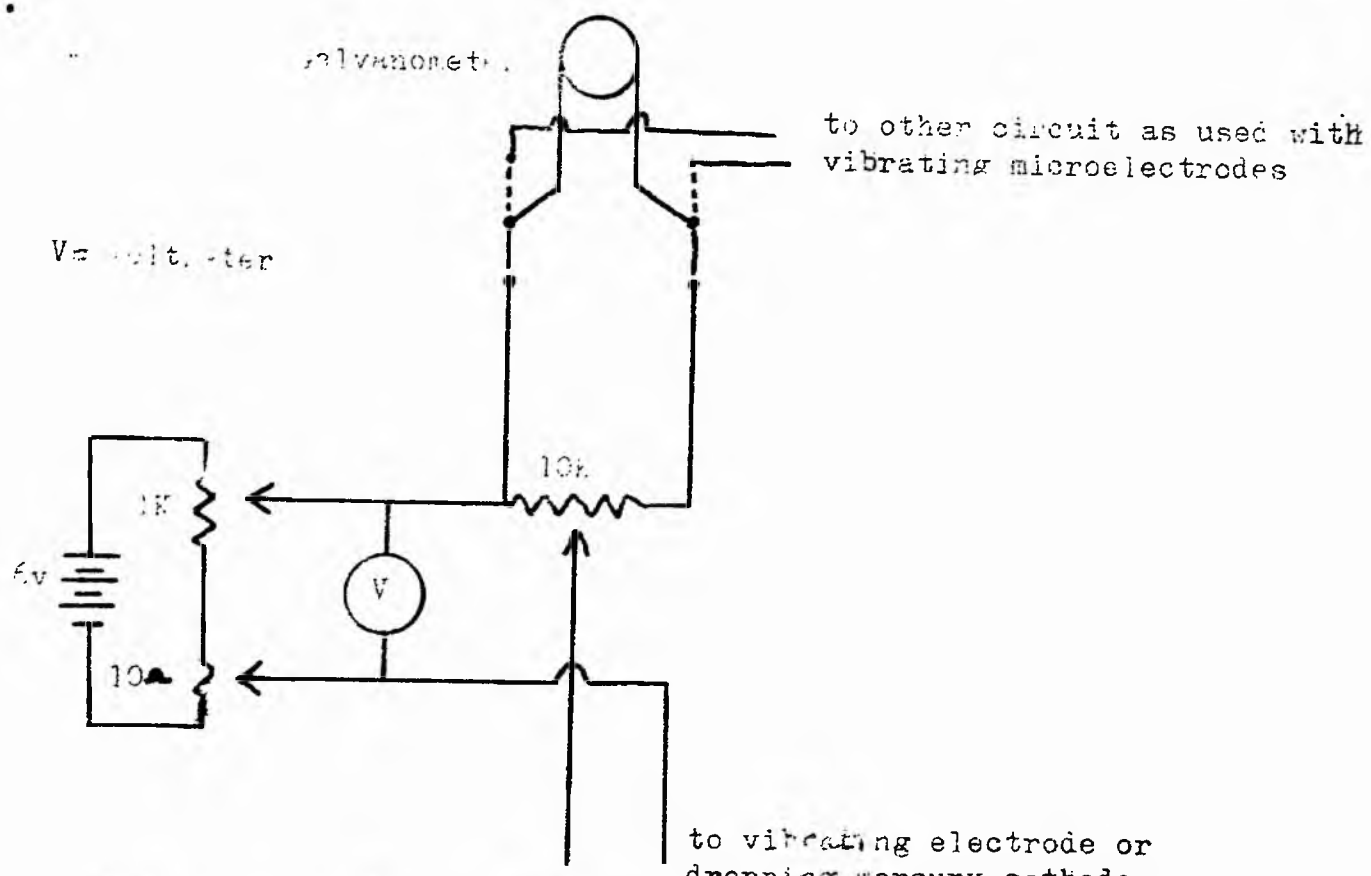


Fig. 19. Circuit used for both vibrating electrodes and the dropping mercury electrode.

A mirror galvanometer was used for measuring the current, as was used in conjunction with the dropping mercury cathode polarograph. The circuit for each electrode was designed so that readings could be taken from either one or the other without disturbing the applied voltage to the electrodes. This was achieved with a double pole, double-throw switch, enabling readings to be taken alternately from either circuit.

6. Calculations

Basically the same calculation was used for all the methods used in measuring oxygen consumption. The quantity of oxygen consumed was calculated from the known values for the volume and temperature of each container, the solubility of oxygen in saline solution and the linear relation between oxygen concentration and diffusion current. From a comparison of values obtained by direct measurement, that is, the galvanometer deflection, with the standard curves previously obtained, fall in oxygen concentration could readily be seen. Thus, from this value and that for the dry weight of the tissue, a value of oxygen consumption in terms of µl./mg. dry weight could be obtained. When using the syringe technique, it must be remembered that the polarographic determination is in terms of oxygen concentration and this must be related to the volume of saline remaining in the syringe.

7. Precautions

When utilising the dropping mercury cathode for determination of oxygen concentration of the solution, care must be taken to keep the capillary tube of mercury free from dirt, or the drops of mercury are neither regular in rate of appearance nor are they

uniform in size. Care must also be taken in filling the syringe to eliminate all air bubbles, as these can obviously create large discrepancies in the results. It was also found that better reproduceability of readings was obtained if the needle was removed when samples were taken from the syringe, thus enabling the saline to pass out both quickly and with less disturbance. If the saline is pushed out of the needle, this allows ample opportunity for gaseous exchange with the atmosphere to take place and this must be avoided.

When using either apparatus I or II, care must be taken to ensure that no air bubbles are present in the fluid. A transparent substance for construction of the chambers is therefore necessary. It was found that small bubbles of air were more readily seen if a bright pin-point of light was shone through the fluid and viewed from the other side, any bubbles appeared as white specks in the fluid. Bubbles of approximately 4 µl. could easily be detected. Any bubbles which are present, however, must be removed before the start of an experimental period. Care must also be taken when clamping off apparatus I, for the clamps must be placed as near as possible to the entry of the inlets and outlets to the chamber to reduce the dead-space, and hence limit the oxygen available to diffuse into the body of the solution from the fluid trapped in these spaces. Such volumes are only about 5% of the total volume of the chambers, but this error could be introduced if the oxygen became available to the tissue.

Oxygen is known to diffuse through perspex, but Leaf and

Renshaw (1957) have shown that using heavy-walled perspex chambers there is no statistical difference between oxygen uptake recorded using either glass or persex chambers. Similarly, there is no significant difference between the results obtained in this study using either the glass syringe or electrodes in perspex chambers. This was further confirmed by conventional Warburg manometry. The use of heavy-walled perspex chambers was therefore justified, especially as in these experiments they were siliconed on their inside surfaces.

8. Sodium-free incubation medium

A choline medium was used for these studies, and had the following composition:-

choline chloride 8.5 g./l., KCl 0.2 g./l., $CaCl_2$ 0.2 g./l., $MgSO_4$ 0.1 g./l., $KHCO_3$ 1.2 g./l., K_2HPO_4 0.2 g./l., and glucose 0.75 g./l. dissolved in deionised water. The pH was adjusted where necessary with KOH or HCl solution to pH 7.8. This medium was stored at 4°C. in 'Pyrex' bottles during use and kept for not more than two days after preparation. A volume of choline medium sufficient for experiments was raised to 25°C. in a water bath prior to the experiments. Bladders were removed from the animals and washed four times for one to three minutes in choline medium to remove any sodium ions, the high concentration gradient existing for sodium would wash out all but bound sodium from the cells. Similarly the paired half bladders used in experiments with sodium medium were washed in sodium saline in order to act as controls against the handling effects which may have occurred during the

choline-medium washing. Half the bilobed bladder was treated in choline medium and half in sodium medium.

9. Thyroxine; triiodothyronine mixture

The oxygen consumption of bladders treated with a mixture of thyroxine and triiodothyronine was measured. Both hormones were added to the serosal surface of the bladder in normal saline at the same time at a concentration of 10^{-6}M and respiration rate was measured, using vibrating platinum electrodes, every 2.5 minutes for sixty minutes.

10. Analogues

3;5;3';5' - tetraiodothyroacetic acid (TETRAC), 3;5;3' - triiodothyroacetic acid (TRIAC), (Glaxo Laboratories Ltd.). 3;5;3';5 - tetraiodothyroformic acid (W. 1489, Lot 00125) and 3;5;3';5' - tetraiodothyropropionic acid (W. 1524, Lot 8) (Warner-Lambert Research Laboratories, Morris Plains, New Jersey) were dissolved in a minimal amount of 0.1 N NaOH before addition of the saline. A similar amount of NaOH was added to the control saline.

11. Hormones

L-Thyroxine (L. Light and Co. Ltd.) and 3;5;3' - triiodo-L-thyronin (Glaxo Laboratories Ltd.) were dissolved in 0.1N NaOH before addition to the saline except when required in choline saline when similar amounts of KOH were used. NaOH or KOH were added to the control media. The hormones were always added to the serosal surface of the membranes, except when used in the syringe technique where, by virtue of the experimental design, the hormones were added to both surfaces at the same time.

C. RESULTS

1. Effect of thyroxine and triiodothyronine on respiration rate of the bladder.

Fig. 20 shows the effect of incubation with 10^{-6}M thyroxine and 10^{-6}M triiodothyronine on oxygen consumption of whole isolated bladders bathed on each side by similar media. Both hormones increase the oxygen uptake, and a linear log. dose response curve is obtained between 10^{-8}M and 10^{-5}M hormone concentration. Respiration of the thyroxine-incubated bladders increases with time over fifty minutes, whereas the oxygen consumption of the triiodothyronine-incubated bladders increases more rapidly over the first twenty minutes, but then falls to a near-normal rate after fifty minutes. In the experiments using the syringe technique the oxygen consumption of the whole bladder before treatment with thyroxine was 1.31 ± 0.08 µl./mg. dry weight/hour (mean ± standard error of twelve experiments) and after forty minutes incubation the rate was 2.56 ± 0.14 µl./mg. dry weight/hour. With triiodothyronine, the oxygen consumption before treatment was 1.00 ± 0.04 µl./dry weight/hour (mean ± standard error of twelve experiments) but doubled after only twenty minutes incubation. Values obtained using the vibrating microelectrode technique showed no significant difference from these results, despite the fact that in the latter case the hormone was only applied to one surface of the bladder - namely the serosal side. In addition, with the bladder as a membrane between the two chambers it was sometimes lightly stretched. Such values are, control tissue 1.33 ± 0105 µl./mg.

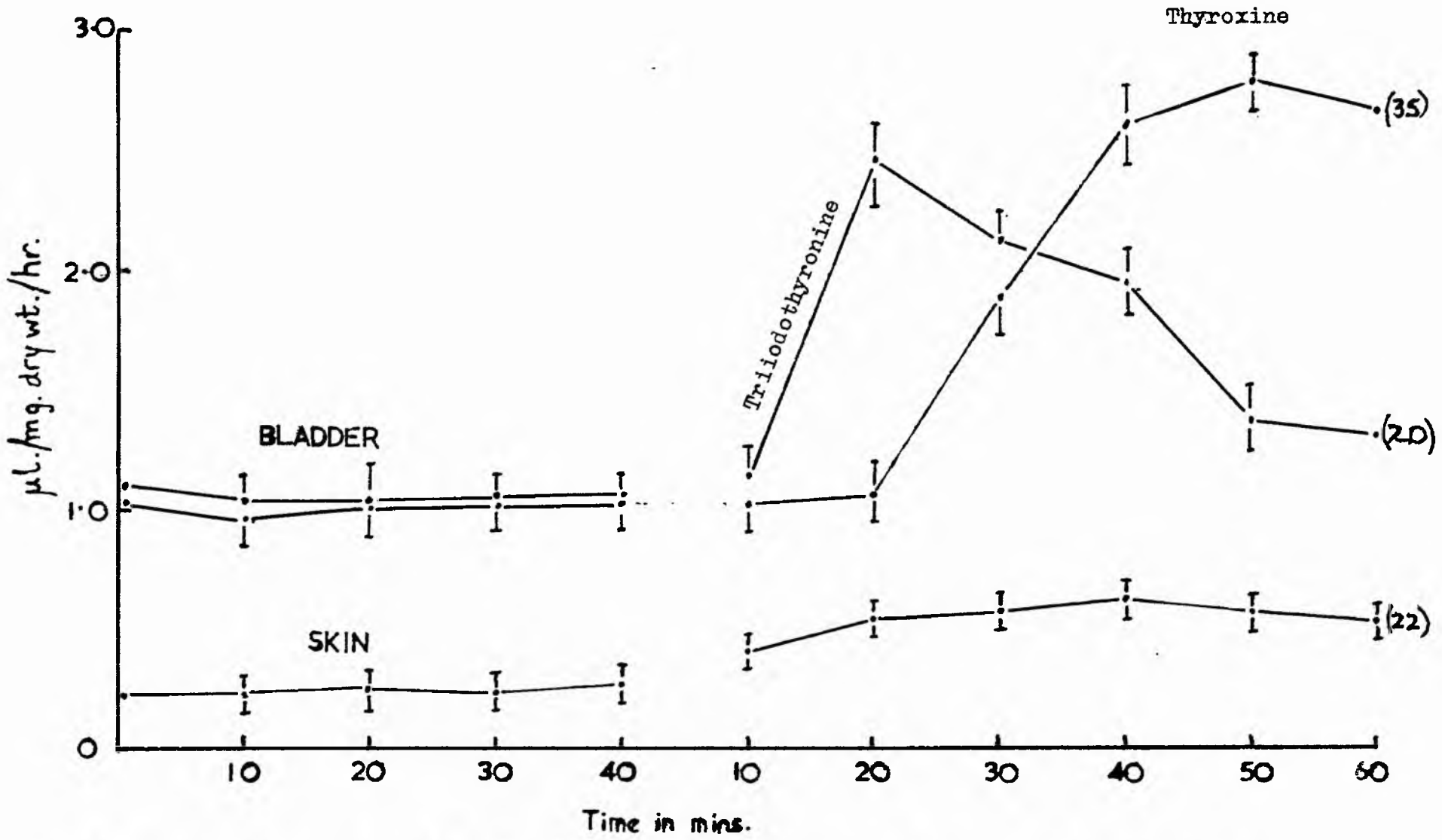


Fig. 20. Effect of 10^{-6} M thyroxine and 10^{-6} M triiodothyronine on oxygen consumption of the isolated bladder, together with the effect of thyroxine on oxygen uptake of isolated skin. All values obtained by the vibrating electrode technique, and are means ± S.E.. Number of experiments in parenthesis.

dry weight/hour (mean ± standard error of thirty-five experiments) and after fifty minutes incubation with thyroxine 2.48± 0.11 µl./mg. dry weight/hour.

2. Effect of thyroxine on the respiration rate of the skin.

Skin respiration was increased by thyroxine in a manner simila to that of the bladder (see Fig. 20). If, however, the oxygen consumption is expressed in terms of membrane area, then the skin oxygen consumption is eight to ten times greater than that of the bladder. This is due to the skin weight per unit area being eight to ten times that of the bladder. Control respiration rates of the skin as determined by the vibrating microelectrode technique were 0.22 ± 0.03 µl./mg. dry weight/hour (mean ± standard error of twenty-two experiments) or 0.067 µmoles/cm²/hour, whilst after fifty minutes incubation with thyroxine the value rose to 0.49±0.07 µl./mg. dry weight/hour (or 0.103 µmoles/cm²/hour).

3. Sodium-free incubation medium.

The absence of sodium ions from the medium normally reduces the oxygen uptake of the isolated bladder of Bufo bufo (Table 10, col. 1), confirming the data of Leaf, Page and Anderson (1959) obtained with Bufo marinus. It was found that only by using paired half bladders could any constant results be obtained and values for oxygen uptake of paired half bladders are given in Table 10, but several experiments were performed to confirm the results. Using the syringe technique it was possible to measure tissue oxygen uptake in saline for a control period, then remove the tissue from the syringe and measure uptake when the bladder was

Table 10. Comparison of Q_{O_2} of isolated toad bladder in presence and absence of sodium whilst incubated in 10^{-6}M thyroxine.

	Half-bladder pairs		Half-bladder pairs		Half-bladder pairs	
	Choline medium	Sodium medium	Choline medium	Choline medium thyroxine	Sodium medium	Sodium medium thyroxine
	1.45	1.67	1.43	2.01	1.61	2.14
	0.95	1.84	1.41	2.12	1.47	2.46
	0.85	1.48	1.41	1.76	1.26	2.97
	1.38	1.64	0.92	2.06	1.10	2.07
	0.72	0.89	1.00	1.70	1.12	2.43
	1.28	1.82	1.21	2.57	1.31	2.52
Mean	1.11	1.56	1.23	2.03	1.31	2.43
Mean of difference of O_2 uptake	0.452		0.806		1.120	
S.E.	±0.106		±0.133		±0.104	

All values are given as ul./mg.dry wt./hr.

immersed in a medium containing thyroxine at 10^{-6}M concentration. Results obtained by this procedure for control half bladders in choline medium, are 1.29 ± 0.09 µl./mg. dry weight/hour (mean standard error of twelve half bladders) and when afterwards immersed in a choline medium containing thyroxine give values of 2.14 ± 0.10 µl./mg. dry weight/hour fifty minutes after application of the hormone, the difference being 0.84 µl./mg. dry weight/hour. Similar experiments with the other half bladder in sodium saline gave the following results, 1.37 ± 0.10, before thyroxine treatment and after fifty minutes incubation with thyroxine 2.38 ± 0.17 µl./mg. dry weight/hour, the difference in this case being 1.01 µl./mg. dry weight/hour. Further to these experiments, some bladders were used as their own controls in both choline and sodium saline. Whole bladders were first washed in choline medium and a control rate of oxygen consumption measured, then removed from the syringe and the uptake measured in choline medium and thyroxine. The bladder was then placed in sodium saline which was renewed every ten minutes for thirty minutes to wash the tissue free of thyroxine and also to allow the membrane to regain lost sodium, as well as allowing the sodium pump to become active again. A control respiration rate was then determined and the bladder was afterwards immersed in a sodium saline containing thyroxine and a further rate measured. Values obtained were, choline medium alone 1.10 ± 0.08, choline medium plus thyroxine 1.92 ± 0.12, (difference; 0.82 ± 0.03); sodium medium alone 1.37 ± 0.10 and sodium medium plus thyroxine 2.43 ± 0.18, (difference; 1.06 ± 0.05) all values being

µl./mg. dry weight/hour, (mean± standard error of eight experiments). These results, therefore, confirm the data already obtained, for in the latter case, all determinations in the different media were made on the same tissue, thus eliminating individual differences between bladders.

Although the absence of sodium from the bathing medium reduces the oxygen uptake of the bladder, in thyroxine-treated bladders an even greater difference exists between the oxygen uptake in the sodium medium and that in the choline medium. The increase in metabolism in choline medium caused by thyroxine represents the general role of thyroxine on enzyme systems, but the proportionally greater increase of respiration in sodium medium suggests that there is, over and above the increase in the basal level, an increase in the metabolic requirements of the enzymes concerned with the sodium transport system.

4. Relationship of oxygen consumption to ionic movement.

The results reported here were obtained from studies of oxygen uptake measured by vibrating microelectrodes, concurrent with electrical measurements of sodium transport across the same bladder. In considering the effect of thyroxine on sodium transport and oxygen consumption of the bladder and skin, it became evident that the oxygen consumption rose at a faster rate than the sodium transport, although following a similar time response curve. The work of Leaf, Page and Anderson (1959) has shown that in the bladder of Bufo marinus, as in frog skin (Zerahn, 1956, 1958; Leaf and Renshaw, 1957) the ratio of equivalents of sodium

transported to oxygen molecules utilised is approximately 18 to 1. During the course of thyroxine action (Table 11) it is 10.7 to 1 during both the thirty minute control and the nought to ten minute period after addition of the hormone at 10^{-6}M concentration. As time increases, this rate decreases, falling to 8.4 to 1 between the thirty to forty minute period. Although both oxygen consumption and sodium transport are increasing, the rate of increase of oxygen consumption is greater than the rate of increase of sodium transport (as can be seen in Fig. 21) and thus the ratio falls. The oxygen consumption is, therefore, being stimulated to a greater extent than that required by the increased metabolic-dependent process of active transport.

If the values for the oxygen consumption in choline medium, obtained per ten minute period after hormonal addition, are subtracted from those obtained in the same time interval using sodium medium, a value of the metabolic requirements for the sodium transport per ten minute period is obtained. In turn, if these values are compared to the increases per ten minute interval in sodium transport given in Table 11, the ratio of 18:1 alters only slightly but remains at or near this value during hormonal treatment. The figures being 0-10 minutes, 15.75:1; 10-20 minutes, 12.0:1; 20-30 minutes, 14.6:1 and 30-40 minutes 13.7:1. It can, therefore, be seen that the rise in oxygen uptake seen in choline medium is related to a stimulation of general enzyme systems, whereas the extra increase found in sodium medium is directly related to the sodium transport requirements. Thyroxine thus

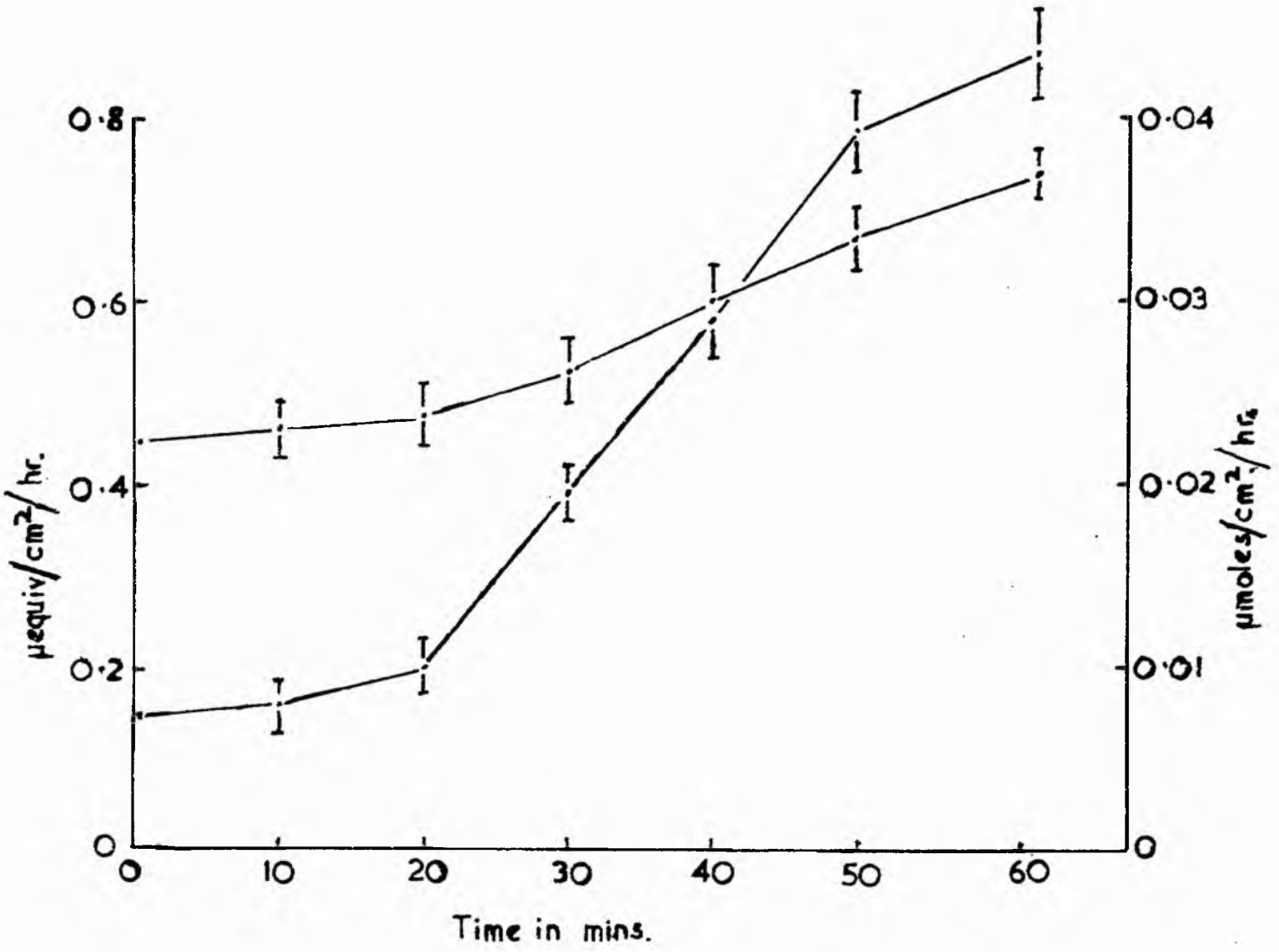


Fig. 21. Thyroxine effect on sodium transport and oxygen consumption of isolated toad bladder. Hormone added at zero time. Data taken from Fig. 12 and Fig. 20.

Table 11. Effect of thyroxine on ion transport and oxygen consumption of the toad bladder.

Time		Sodium transport (μeq/cm^2/hour)			Oxygen consumption (μmoles/cm^2/hour)			
		Before	After	Na	Before	After	O_2	Na/O_2
0-10 mins.	1	0.610	0.622	0.012	0.0067	0.0075	0.0008	15.00
	2	0.475	0.500	0.025	0.0067	0.0080	0.0013	20.00
	3	0.421	0.438	0.017	0.0079	0.0085	0.0006	28.00
	4	0.377	0.389	0.012	0.0071	0.0078	0.0007	17.14
	5	0.400	0.414	0.014	0.0087	0.0094	0.0007	19.43
	6	0.394	0.415	0.021	0.0077	0.0088	0.0011	18.73
Means		0.446	0.463	0.0167	0.0074	0.0083	0.0009	19.717
10-20 mins.	1	0.622	0.649	0.027	0.0075	0.0108	0.0033	8.24
	2	0.500	0.584	0.084	0.0080	0.0114	0.0034	24.71
	3	0.438	0.480	0.042	0.0085	0.0117	0.0032	13.13
	4	0.389	0.430	0.041	0.0078	0.0100	0.0022	18.64
	5	0.414	0.450	0.036	0.0094	0.0133	0.0039	11.94
	6	0.415	0.463	0.048	0.0088	0.0113	0.0025	18.23
Means		0.463	0.509	0.046	0.0083	0.0114	0.0031	15.81
20-30 mins.	1	0.649	0.725	0.076	0.0108	0.0210	0.0102	7.45
	2	0.584	0.654	0.070	0.0114	0.0222	0.0108	6.48
	3	0.480	0.556	0.076	0.0117	0.0203	0.0086	8.84
	4	0.430	0.492	0.062	0.0100	0.0154	0.0054	11.48
	5	0.450	0.516	0.066	0.0133	0.0199	0.0066	10.00
	6	0.463	0.551	0.088	0.0113	0.0194	0.0080	10.86
Means		0.509	0.582	0.073	0.0114	0.0197	0.0083	9.19
30-40 mins.	1	0.725	0.809	0.084	0.0210	0.0291	0.0081	10.37
	2	0.654	0.838	0.184	0.0222	0.0341	0.0119	15.51
	3	0.556	0.632	0.076	0.0203	0.0352	0.0149	5.11
	4	0.492	0.556	0.064	0.0154	0.0219	0.0065	9.85
	5	0.516	0.572	0.056	0.0199	0.0300	0.0101	5.51
	6	0.551	0.605	0.054	0.0194	0.0326	0.0132	4.09
Means		0.582	0.669	0.086	0.0197	0.0305	0.0108	8.41

appears to invoke an additional increase in the metabolism, as measured by oxygen consumption, above that required for the thyroxine-induced increase in active transport. A conclusion which is also apparent from the experiments using sodium-free incubation media. The effect of thyroxine upon the respiration rate when in this medium is to increase the oxygen uptake, despite the lack of stimulation of active sodium movement.

5. Thyroxine: triiodothyronine response.

Both hormones when added together cause a rise in respiration rate within the first twenty minutes and then falls slightly before increasing again (Fig. 14). The initial rapid rise is similar to that obtained in triiodothyronine alone, and the latter part of the curve is similar to that obtained with thyroxine alone. A similar biphasic response is obtained when water loss and short-circuit current are measured.

6. Analogues

None of the analogues used caused any increase in oxygen consumption of the bladder, although readings were taken from each side of the membrane at intervals of one minute. Thus the effect tetraiodothyroacetic acid and triiodothyroacetic acid on increase of water movement down an osmotic gradient across the bladder cannot be accounted for by an increase in metabolism.

D. DISCUSSION

Few *in vitro* experiments have been reported on the effect of

thyroidal hormones on respiration of adult amphibian tissues, b both Ahlgren (1925) and Haarman (1936) observed an increase in oxygen uptake in the isolated muscle of the frog upon incubatio in thyroxine solution. The present experiments, however, show that both thyroxine and triiodothyronine, when added to a physio logical saline solution bathing either an isolated toad bladder piece of skin, increase the oxygen uptake of these tissues, indi cating that the thyroid hormones may be controlling oxidative metabolism in amphibian tissue as they do in mammals.

Comparison of Figs. 3, 12 and 20 indicates that changes in water permeability, short-circuit current (active sodium transpor and respiration, as influenced by thyroxine and triiodothyronine, follow the same time course. This is well illustrated in the ca of triiodothyronine where maximal rates of all the parameters are obtained in twenty minutes, all falling to control values within an hour. It does not appear from these studies on the toad bladder and skin, however, that triiodothyronine, when administere in equimolar concentrations induces a greater maximal oxygen uptake than thyroxine. This is in contrast to the results of <u>in vitro</u> and <u>in vivo</u> treatment of mammalian tissue, where triiodothyronine produces not only a more rapid but also a greater maximal response th thyroxine (Gross and Pitt-Rivers, 1953; Barker, 1956). The application of both hormones together on the serosal surface of the bladder results in a biphasic curve, showing a distinct relationship to the curve which would be found after superimposing the separate graphs of thyroxine and triiodothyronine response.

The curve obtained, however, shows a shift of the 'thyroxine' part of the curve towards the point of addition of the hormones, indicating that the hormones can act together. Presumably triiodothyronine is influencing either the penetration of thyroxine into the cells or its attachment to sites on the cell surface. In the experiments described here the increase in respiration obtained is probably a true indication of enzymic activity, for although concentrations of L-thyroxine between 10^{-4}M and 10^{-3}M have been shown to uncouple oxidation from phosphorylative processes (Dallam and Howard, 1960), such concentrations are far higher than the concentrations used in the present experiments.

Replacement of sodium by choline in the medium reduces the oxygen consumption of the toad bladder and the increase brought about by thyroxine in such a medium is less than that found when the bladder is immersed in a sodium medium. Relationships may now be drawn between ionic transport mechanisms and oxygen consumption under thyroxine treatment.

The results may be compared with the data previously reported on vasopressin action, for this has been fully examined during recent years. General cellular metabolsim appears to be hardly affected by vasopressin (Leaf, Page and Anderson, 1959; Goodfriend and Kirkpatrick, 1963) but it increases the active transport of sodium, which is an energy-dependent process (Leaf, Anderson and Page, 1958). The increase in respiration brought about by vasopressin in sodium medium is of the order of thirty percent (Leaf, (1961), whilst there is little or no increase in the first hour

after addition of this hormone to bladders incubated in a sodium-free medium. The present results show an approximately hundred percent increase of oxygen uptake after addition of thyroxine to sodium medium and approximately eighty percent increase in sodium-free medium. Thus thyroxine, unlike vasopressin, does not appear to be restricted in its control of metabolic processes. This effect is probably due to the fact that thyroxine neither directly affects pore size nor is it directed specifically to sodium kinetics, but has a generalised role in stimulating enzyme systems. Pitt-Rivers and Tata (1959, pages 103 to 105) have tabulated many biochemical observations on enzyme systems, both *in vitro* and *in vivo*, which show a lack of specificity of action of thyroid hormones.

Net transport of sodium across both isolated bladder and skin of the toad is increased by thyroxine and from the present study it can be seen that the increase in oxygen consumption is far greater than that utilised by the energy-dependent carrier system. The relationship between sodium transport and oxygen consumption has been shown to be steady even under conditions of stimulated sodium transport, for the membrane uses one equivalent of oxygen for every eighteen equivalents of sodium moved (Zerahn, 1956; Leaf and Renshaw, 1957). The latter authors have demonstrated that this relationship holds when frog skin is stimulated with vasopressin and, indeed, shown that under these conditions the ion transport is increased to a greater extent than is oxygen consumption. As this is not the condition in thyroxine-treated membranes, it is evident that since both sodium transport and oxygen consump-

tion are increased, the observed fall in the sodium transported: oxygen consumed ratio is caused by greatly increased oxygen consumption.

In choline saline there is no sodium transport in either the control or thyroxine-treated state, but in the latter condition there is increased tissue respiration. When immersed in sodium saline the respiration rate is slightly higher than when in sodium-free medium and when stimulated by thyroxine, the difference between the two respiration rates, in sodium-containing and sodium-free media, increases. It is evident from the results that this amount of oxygen, that is the difference between the two respiration rates (even when treated with thyroxine), is that required by the sodium transport system. The results obtained in non-stimulated conditions show that the ratio is approximately 18:1 but, as the results show, deviations do occur when the values are worked out for treated period and this is presumably due to the fact that the data used in these calculations is taken from different experiments. Despite these slight variances it appears that there is a rise in general metabolism, that is, the increase found in choline medium plus thyroxine, as well as an additional increase as a result of increased sodium transport, that is, the difference between the respiration rates in sodium and choline media. Thyroxine seems therefore to effect both the enzyme systems involved in active sodium transport and also those concerned with general cellular metabolism.

The present results, therefore, clearly indicate that thyroxine

and triiodothyronine increase the respiration rate of two amphi tissues *in vitro*, namely the bladder and skin. Although the m branes are known to be intimately concerned with active sodium port, it has been shown conclusively that the thyroid hormones a the metabolism of the cell in addition to stimulating the moveme of salt. The increase of oxygen consumption reported here has confirmed in this laboratory by Thornburn and Matty (1963) who, using the more conventional Warburg technique for measuring oxyg uptake, found stimulation of bladder and skin after application o thyroidal hormones and in addition, detected increases in heart an skeletal muscle respiration. These reports confirm those of Ahl (1925), who measured the oxygen uptake utilising the method of re duction of methylene blue as an indication of oxidatibe capabilit of tissue, and of Haarman (1936) who also found an increase in res piration of frog skeletal muscle respiration after addition of thy xine to the bathing medium.

The report of Hickman (1959) is of particular interest in thi respect, for he has shown that the increased metabolic rate of fish in hypertonic media is correlated with increased thyroid secretion and, further, both these effects are related to the osmoregulatory capacity of the animal. The quantitative differences in active ion transport would appear to be the origin of the greater demands for thyroid hormone when the fish is transferred to salt water. He suggests that a possible point of action of thyroid hormone would be a direct effect on the cells performing the osmotic work. Both metabolic rate and the thyroid activity are significantly less in

fresh water than in sea water, but this is not inconsistent with the theoretical thermodynamic energy demands for osmoregulation in the former medium. The results are related to the demands by the cells performing active transport and, although the experiments were performed in a different group, they do offer some comparison to the present work. Although the animals are living in a different environment to that of Amphibia and are faced with different problems, osmoregulation is a fundamental process in both groups. In the present work thyroxine has been shown to cause increased ion transport of salts across membranes, together with an increase in oxygen uptake. The latter effect being a result of the direct relationship between the amount of salt transported and the energy (in terms of oxygen consumption) needed for the process (Zerahn, 1958; 1961). In the light of the present work, however, it appears that the hormones alter general metabolism as well, thus implicating the thyroid hormones not only in an osmoregulatory role but in addition suggests that thyroxine controls cellular metabolism.

The present results show that if readings are not taken at frequent intervals during *in vitro* work then it is possible that the effect of the thyroid hormones on respiration in the tissue used may pass unnoticed. It may well be that certain investigations on *in vitro* effects of the thyroid hormones in the past have failed to make sufficient observations and therefore have only an approximation of the correct result. Doubt as to the validity of certain of the earlier reports is possible, on the basis that the thyroid preparations used may have been impure and contaminated with sub-

stances capable of stimulating respiration. The present work, ever, together with other recent work using synthetic hormone (1962; Halmolsky, Michel, Carnicero and Roche, 1963) confirms th data obtained in the early periods of investigation of *in vitro* thyroxine effects. There is also a great divergence in the met of measuring the metabolism of the tissue and although the Warbu technique has been shown to be extremely sensitive, it is perhap significant that the workers who have observed increases made mo frequent observations whereas the negative reports originate fro results taken at larger intervals of time after hormonal addition

Readings taken every thirty minutes would only give a slight variation from the control levels at not more than three points a the most, on the basis of the present experiments, and this could easily be mistaken for variance in the tindividual pieces of tiss This critisism of *in vitro* investigations applies to those made i all classes of vertebrates where low concentrations of hormone hav been investigated over long periods of incubation. It is worth noting that Hamolsky, Michel, Carnicero and Roche (1963) also find that the effect of thyroxine on the oxygen consumption of isolated horse leucocytes becomes apparent approximately thirty to forty minutes after addition of the hormone. ork on mammalian tissue has the disadvantage that the respiration rate is found to fall of even over five minute intervals, but nevertheless responses have been obtained which indicate a sustaining, rather than a stimula- tory role of metabolism in these tissues (Barker, 1956; Grief and Moroney, 1950). Some of the negative *in vitro* work on the effect

of thyroid hormones on metabolism may be related to the work of Weinstein and Lein (1957) who demonstrated a dual effect on the isolated rat diaphragm. They found an increase in respiration at low concentrations whilst at concentrations of thyroxine above $10^{-4}M$ a depression of oxygen consumption occurred, a concentration where uncoupling of phosphorylation also occurs (Dallam and Howard, 1960; Lehninger, 1956). Supporting this view is the work of Martius and Hess (1951) who showed that thyroxine, between $10^{-4}M$ and $10^{-5}M$ concentration suppressed aerobic respiration, whereas at lower concentrations a stimulation was found. Many authors in the past have used very high doses but these may have a pharmacological rather than a physiological effect and many of the negative reports, both *in vivo* and *in vitro*, may be due to this effect.

Although there are many negative reports on *in vitro* thyroxine effects on tissue respiration, quite a number of them may be regarded as unphysiological due to the factors mentioned above. There is evidence, however, that the thyroid hormones affect metabolism in the lower vertebrates as they do in mammals, as several *in vitro* and *in vivo* reports indicate. The review of Leloup and Fontaine (1960) presents evidence from a number of interesting reports which indicate that the thyroid may be implicated in respiratory exchanges in the lower vertebrates. There is a more elevated thyroid secretion in active species as well as in trout in stream(which also consume more oxygen) and increases of secretion in smolt and parr which again corresponds to an increased oxygen uptake. One interesting finding is that the oxygen requirements

and thyroid secretion show a concurrent decrease in the aestivatin lungfish (Smith, 1930). Muller (1953) on fish, and Donoso and Trivelloni (1958) and Warren (1940) on toads and frogs respectivel have all demonstrated a metabolic effect on whole animals in these classes of vertebrates following either injection or feeding with thyroxine, triiodothyronine or thyrotropic hormone. Galton and Ingbar (1962a), however, failed to find any effect of either thyroxine or triiodothyronine on the respiration rate of adult frogs, or any metabolic effects on tissues isolated from these animals. In view of the large body of contradictory data in the literature, it is unlikely that any single study can determine conclusively whethe Amphibia are responsive or not to thyroid hormones. The lack of metabolic activity of the analogues investigated in this study tend to suggest that the periphially 'active' forms of the thyroid hormones are, in fact, thyroxine and triiodothyronine. The other forms found within the cell may be simply degradation products formed as a result of their metabolism within the cell. These results in the amphibia support the idea that the principal hormones released from the thyroid are the active forms, in a manner similar to that suggested for mammals by Tata (1961) and Pitt-Rivers and Tata (1959).

Under the present experimental conditions, though, several indications were found that thyroxine and triiodothyronine affected enzyme systems within the cell, including those concerned with general metabolism and those directed towards active sodium transport. The latter process has been shown to be intimately connected with enzyme systems within the cell (Maffly and Edelman, 1963;

Canessa-Fischer, Edelman and Davis, 1962), and such a simultaneous effect on these two parameters is not unexpected.

SECTION V.

Thyroxine effect on different aspects of ion balance in the toad.

A. INTRODUCTION

The previous work on the effect of thyroidal hormones on the isolated toad bladder and skin demonstrated that these hormones increased active sodium transport across the two membranes and, in addition, increased the passive permeability of the isolated bladder to water, either in the presence or absence of an osmotic gradient. Following this work it was decided to investigate the ion and water balance of the whole animal in response to thyroxine. The thyroid hormones have also been shown to influence the metabolism of at least three tissues, namely skin, bladder and muscle. The effect of thyroxine upon body weight of the toad during treatment was, therefore, followed in this study, this being used as an index of metabolic activity of the hormone.

Dehydrated toads are capable of very great uptake of water when returned to water (Ewer, 1952b), whilst the frog triples its water uptake under similar conditions (Levinsky and Sawyer, 1953). It has been known for many years that Amphibia can remove salt from fresh water and similar dilute solutions, into the body (Krogh, 1937) and also that the ions are moved in against an intense concentration gradient (Krogh, 1938). Indeed, this was demonstrated *in vitro* by Huf (1935, 1936) who, although measuring only chloride movement in open-circuited skin, concluded that there was an active shift of sodium chloride from mucosal to serosal surfaces. Later, Ussing and Zerahn (1951) showed that there was

an active movement of sodium ions which created a passive movement of chloride ions across the skin from the mucosal to serosal surfaces. The bladder of Amphibia is also capable of removing ions from the urine and transporting them into the blood, as the *in vitro* studies of Bentley (1960) and Leaf (1961) have shown.

Emphasis has been placed upon the neurohypophyseal hormone regulation of ion and water balance in Amphibia. Injection of these hormones, particularly those related to vasopressin, has been shown to influence salt uptake of frogs (Heller, 1957; Sawyer 1961a,b) and of the axolotl (Jørgensen, Levi and Ussing, 1949). These principles have also been shown to influence water uptake through frog skin *in vivo* (see Heller, 1945) and also to slightly affect the rate of water excretion of the toad (Jørgensen, 1950) although they have little effect on the excretion of the frog (Levinsky and Sawyer, 1953). Ewer (1952a) was the first to demonstrate that neurohypophyseal extracts caused, or accelerated, the reabsorption of urine from the bladder of *Bufo regularis*. That the water was resorbed across the bladder wall and not absorbed after regurgitation into the cloaca was conclusively shown by Sawyer and Schisgall (1956). The antidiuretic properties of these posterior pituitary principles in other animals are well known (Heller, 1956; Sawyer, 1961b), the hormones causing an increased renal resorption of water. An increased sodium loss is known to occur, however, in both amphibia and mammals (see Heller, 1950) and also in cyclostomes (Bentley and Follett, 1962). The picture in teleosts, however, is not clear, for Holmes (1959)

found that vasopressin depressed the renal excretion of sodium in salt-loaded trout, while Maetz and Julien (1961) found that oxytocin increases sodium uptake in the goldfish. In addition, the influx of water in the killifish was reduced by pitocin and high doses of pitressin, and a similar effect was noticed on the water outflux. Sodium uptake was stimulated by both the hormonal mixtures, but only pitocin decreased sodium loss through the gills (Meier and Fleming, 1962). It must be remembered that differences in ionic regulation exist between the fish and amphibia and these are basic in interpreting hormonal effects. The same hormone may exert an effect in different groups by acting on different effector sites and, although appearing to differ in their effects because of the different requirements of the animal, may, in fact, effect similar basic mechanisms.

Many hormones have been shown to affect ion balance in different groups (for a review of mammalian work see Kruhøffer, Thaysen and Thorn, 1960). In lower vertebrates, Holmes (1959) found that desoxycorticosterone acetate increased sodium excretion from the salt-loaded rainbow trout, a result similar in nature to that obtained by Sexton (1955) on goldfish. Holmes and Butler (1963), after administration of cortisol, corticosterone and aldosterone to trout, also found a loss in plasma sodium and a rise in muscle sodium. Angerer (1950) noted that adrenalectomy of frogs caused a decrease in potential difference across the skin which may be taken as a measure of decreased salt transfer, while Jørgensen (1947) found that the sodium loss via the kidneys and skin is in-

creased after adrenalectomy.

The thyroid hormones have, however, also been shown to affect ionic regulation in different groups of animals. Fontaine (1956) has reviewed the evidence for thyroidal control of ionic movement in fish and concluded that the action of the gland is to favour the passage of fish into hypotonic media, probably by causing retention of chloride and the elimination of water. In the trout, for example, thyroxine augments chloride excretion upon transference from sea-water to distilled water (Baraduc, 1957), thereby assisting in the osmoregulatory process. Smith (1956), however, although finding that thyroxine injection into brown trout raised the salinity tolerance, showed that the doses required appear to be above the normal physiological level. The work of Hickman (1959) has shown that the flounder undergoes a change in electrolyte balance when immersed in different salinities and correlated with this are changes in thyroid activity, which implies that the thyroid secretions are involved in this osmoregulatory mechanism. The effect of thyroid hormones on diuresis in mammals is well known (Brull, 1940; Gaunt, 1944; Pitt-Rivers and Tata, 1959), but the effects on ion regulation are little known. The recent work of Stephan, Jahn and Maetz (1959a,b) has tended to offset this, however, with their reports that hypothyroid rats show a decreased renal tubular resorption of water and ions. Thyroxine injection was found to restore the ion and water balance to normal values.

There is little literature concerning the effects of thyroxine

on ion and water balance of the adult intact toad, but some data exists which has originated from studies of the physiology of moulting in which thyroxine has been shown to have a role.

The thyroidal control of moulting of Urodeles has recently been investigated thoroughly by Clark and Kaltenbach (1961) and that of Anura by Jørgensen and Larsen (1961). Both studies revealed that thyroxine exerts some control of moulting, although differing quantitatively in each group. It appears that thyroidectomy causes a cessation of moulting in Urodela, whilst replacement of thyroid secretion by thyroxine feeding or injection results in a resumption of the moulting cycle (Adams and Gray, 1936; Taylor, 1936). In addition, implantation of thyroxine pellets into the partly neotenous Amphiuma causes an increase in the frequency of shedding of the epidermis over that of control animals (Kobayashi and Gorbman, 1962). In the Anura, however, thyroidectomy does not completely inhibit the moulting cycle, although Ungar (1933) showed that thyroxine injection caused a sloughing of the outer skin layer in Bufo arenarum. Thyroxine treatment, without modifying the process of moulting, was found to decrease the inter-moult period (Jørgensen and Larsen, 1960, 1961). Therefore, although thyroxine may not exert such a complete role in moulting in Anura as it does in Urodeles, it nevertheless exerts some control over the process despite the possibility that it is acting syngergistically with other hormones.

Jørgensen (1949) noted that when toads were moulting both water uptake and sodium influx were enhanced. Koefoed-Johnsen

and Ussing (1949), whilst studying the effect of corticotrophic hormone (ACTH) on the ion balance of the axolotl, made a few observations on the effect of injected thyroxine on salt uptake. Thyroxine increased salt uptake, the effect being quantitatively less than that brought about by ACTH. Also, Levinsky and Sawyer, (1952) have shown that thyroxine causes an increase in body weight, and hence water content, of cloaca-ligatured Rana pipiens, a finding which supports the observation by Heller (1930) who found an increase in water uptake of frogs after thyroxine treatment. In contrast to these findings, which all implicate the thyroid hormones in both water and ionic regulation in the Amphibia, Ewer (1951), after feeding Bufo regularis on 0.25 mg. thyroxine per day for four days, could find no significant difference between the water uptake of the treated and that of the control animals. It must be pointed out though, that this dose is an exceptionally high one, even when given orally and the results must perhaps be regarded as unphysiological.

It appears to be a disputable point whether or not there exists a metabolic effect of thyroxine in adult Amphibia. Warren (1940) found a decrease in body weight of frogs over a period of ten weeks when fed thyroxine twice weekly during this time, this being indicative of a metabolic response, while Taylor (1939) showed that the respiration rate of newts, into which two extra thyroids had been implanted, rose to 187% of the normal animals, which is greatly indicative of a metabolic effect of the thyroid secretions. A direct response on metabolism in terms of oxygen consumption

has recently been seen by Donoso and Trivelloni (1958) after injection of toads with physiological doses of either triiodothyronine or thyroxine. Henschel and Steuber (1951) also found that thyroxine causes an increase in metabolism of frogs, as measured by the calorie production per gram per twenty four hours. Groups of frogs were used to eliminate individual differences and increases were obtained in summer frogs kept at 19°C. A few years prior to this latter observation, Gayda (1922) found that the feeding of thyroid extract produced no effect on the oxygen consumption of the toad. For several years after Warren's work no investigations were performed on whole animal respiration until that of Donoso and Trivelloni in 1958, but recently Galton and Ingbar (1962 a,b) have investigated the metabolic effect in both frogs and a neotenous urodele. They found no effect of thyroxine, or of several analogues, on whole body oxygen consumption of the frog, nor on the oxygen uptake of either slices or homogenates of liver or muscle when this was measured after removal of the tissue from pre-treated animals. Donoso (1960), however, found highly significant increases in the respiration of exised tissue from pre-treated toads. The <u>in vitro</u> work presented in this study (see section IV) indicates that both thyroxine and triiodothyronine affect the metabolism of exised tissues when added <u>in vitro</u> and therefore, in the present study weight changes were followed as these, particularly in starved animals, may be indicative of any metabolic effect of the hormones.

B. MATERIALS AND METHODS.

1. Animals.

The mature Bufo bufo used in this study were obtained from a commercial source (L. Haigh and Son, Surrey), and immediately upon arrival were put into a constant temperature room. They were starved for twenty one days prior to the beginning of the experimental procedure. For fourteen days of this period the animals were kept at 12°C. and two days prior to the operative procedure were placed in a constant temperature room at 15.5°C. The animals were not fed during any time of the experiment and appeared in good health at the end of the experimental period as judged by the appearance of the skin and firmness when held.

2. Operative Procedure.

Animals were anaesthetised lightly with ether and then held in place on a cork board with elastic bands. A polythene catheter was inserted into the cloaca and sewn into place. In some preliminary experiments a polythene cannula was ligatured into the abdominal vein, which lies under the subcutaneous muscle layer and runs from the posterior region up to the liver. This procedure can be readily performed in the toad, for there are two returns of blood to the heart, one via the vein mentioned and the other via the kidneys, thus even after blocking one of venous returns blood can still return to the heart via the other system. Due to a failure of the temperature control, however, these animals died and it was decided for the present experiments not to insert the cannula, which was to be used as a point of injection of solutions.

3. General Procedure.

After recovery from the anaesthesia the animals were placed in wire cages, made from ½ inch mesh wire gauze, which were 6 cms. diameter and 5 cms. high and had a lid cut into the top. The cages were then placed into 230 ml. plastic containers, in which a hole had been drilled into the side near the bottom. Through this hole a rubber tube passed and this was fitted onto the catheter, this rubber tube could readily be removed from the catheter when the animal was weighed. The tube was greased, where it passed through the hole in the plastic container, thus making the joint watertight. Urine flowed via the catheter and rubber tubing into a polythene bottle. The cages were all placed on a wooden stand and the polythene collecting bottles stood underneath this with the rubber tubes passing into them. 100 ml. of tap water (approximately 500 μEq./l. Na and 0.5 μEq./l. Ca) at 15.5°C. covered the body of the toad except for the head. Air, supplied from an aquarium pump, was bubbled first through a water bottle and thence into the bathing fluid via fine polythene tubing which was sealed at one end and pierced a number of times with a pin. Since toads are known to respire cutaneously when submerged in water, aeration possibly helped to maintain the toads in good condition. Immediately after catheterisation and for two days prior to the beginning of the control period, the toads were bathed in tap water containing 150 units per ml. penicillin and the sewn tissue was seen to have healed. During all pre-treatment, control and thyroxine periods the animals were subjected to days of twelve

hours light and twelve hours darkness.

At twelve-hourly intervals 20ml. samples of the bathing fluid were taken along with the total urine excreted during the previous twelve hour period and a 20ml. sample of the tap water. The weight of the animal plus the cage was recorded. In the case of urine sampling, care was taken to empty the rubber tube in order to reduce dead-space effects, which could be appreciable since there was a total space of approximately 1 ml. in the tube and this volume of urine would not normally be seen until the next sample twelve hours later. Thus it was essential in order to obtain satisfactory values for both urine output and weight (for some urine would normally remain in the catheter) to empty the tube when the urine sample was taken. The tap water in which the animals had been kept for the previous twelve hour period was replaced with fresh water which had been stored in large containers at the beginning of the experimental period. The replacement solution, therefore, had the same composition as the initial water throughout all the experiment and also had the added advantage of being at the temperature of the room.

For weighing, the animals were weighed to the nearest 20 milligrams after being allowed to drain off adsorbed water for ten minutes. In the results, the cage weight was subtracted from all other weights. This procedure was performed for three days before the control period was started in order to adapt the animals to the regime, and thereafter throughout the experiment. As a check that any effects which may be obtained could be due to thyroxine,

a further control period of six days was performed after the thyroxine treatment. Each day, at the first sample period, the toads received injections of either 0.1 ml. isotonic saline (see II,B,2) or 0.1 ml. of saline containing 25 µg. thyroxine. Injection was made into the fleshy pad between the external openings of the nares, with an extremely fine hypodermic needle (2½/10 needle; Harris, Birmingham). Injections of saline were made for seven days and then thyroxine injections were made once a day for seven days, this was followed by the later control period of six days during which control saline injections were again given. No handling diuresis was observed during the injection or the weighing procedure and there were no visible signs of distress during any part of the experiment.

4. Analytical Procedure.

Immediately after the samples were taken they were analysed for ion content. Sodium was analysed using an EEL flame photometer and chloride using an EEL chloride meter. The flame photometer was adjusted for each determination with the standard fluid used (either 1 mEq./l. or 10 mEq./l.) and with suitable dilutions of these standards in order to check the calibration curves obtained prior to the experiment. The chloride meter was also checked against known standards and blank determinations carried out using double distilled water twice daily.

5. Precautions.

It is imperative that the animals should not be able to move freely inside the wire cages and thus free the catheter from the

rubber attachment. The size of cage used in these experiments was found to be such that the animals were restricted in their movement, but were able to alter position slightly. In no instance did any animal pull off the rubber connecting tube. As has been pointed out, it is essential to ensure that the rubber tube is emptied of the urine which is found in the undrained tube, otherwise false readings will be obtained and these may tend to mask any effects which may be produced by the treatment. Care must be taken when handling the cages, for mere physical disturbance of this kind is known to produce a release of urine and this would not represent the natural course of events. This was not observed in any of the present experiments.

6. Calculations.

The total amount of each ion lost into or gained from the bathing fluid was calculated from the amount in the tap water when added to the animal and the amount remaining at the end of the twelve hour period. Although in such experiments no separate estimation of the intake and loss through the skin can be made, net changes in the bathing fluid can, however, be measured. Thus the net ion balance can be used as an index of hormonal effect although having the limitation it cannot distinguish the separate effects of thyroxine on influx and outflux.

7. Hormone.

L-thyroxine (L. Light and Co. Ltd) was dissolved in a minimal amount of 0.1N. NaOH before addition to the saline medium to give a final concentration of 25 µgm./0.1 ml. Fresh hormonal solution

for injection was made up daily.

C. RESULTS.

1. Effect of thyroxine on salt transfer through the skin.

Table 12 shows the changes of sodium and chloride content in the bathing fluid before, during and after injection of 25 ugm. thyroxine per toad per day. A net loss of sodium and chloride from the toad to the bathing fluid occurred during the control period. Each injection of saline, however, introduced 15 uEq. of each ion into the body. As can be seen in the Table the amounts of both sodium and chloride lost to the bathing fluid during the initial control period of seven days are consistent over twelve hour periods. The constancy of the amount of each ion lost during these periods is an indication of the adaptation by the animal both to the experimental conditions and the regime employed. There was a slight net loss of sodium ions (loss greater than amount injected plus absorbed) and a net loss of chloride (loss greater than the amount absorbed plus that injected) during the control period, although the amount injected is not taken into account in the Table. To convert the values given to the net value 7.5 μEq. must be added to each twelve hour value (15 μEq. injected every twenty four hours After four days treatment with thyroxine the almost stable (i.e. equal amounts lost and gained) sodium values became gross net gains the values being approximately 10 μEq. per twelve hours. After

of the toad Bufo bufo.

		Bathing Fluid		Urine		Urine	Weight
		Sodium	Chloride	Sodium	Chloride	Volume (ml)	(gms.)
Pre Thyroxine treatment	1	-11.2 ± 2.4	-34.4 ± 4.3	11.4 ± 1.9	2.9 ± 1.6	4.9 ± 1.9	53.8 ± 4.3
		-12.6 ± 1.8	-34.8 ± 3.8	13.1 ± 2.3	10.5 ± 2.4	5.2 ± 1.6	54.1 ± 4.2
	2	-12.5 ± 2.2	-38.3 ± 3.9	11.3 ± 1.7	7.6 ± 1.7	6.2 ± 1.8	54.8 ± 4.3
		-11.0 ± 1.9	-29.1 ± 3.5	16.3 ± 2.7	5.4 ± 2.1	6.1 ± 2.1	55.4 ± 4.5
	3	-9.5 ± 2.1	-33.6 ± 2.7	8.0 ± 1.3	3.6 ± 1.9	8.7 ± 2.0	55.1 ± 4.0
		-8.9 ± 1.7	-30.6 ± 3.8	10.5 ± 1.7	2.6 ± 2.0	8.3 ± 1.8	55.8 ± 3.7
	4	-10.2 ± 2.5	-31.0 ± 3.5	12.3 ± 2.1	6.9 ± 1.9	6.8 ± 1.9	54.0 ± 3.5
		-15.1 ± 2.8	-38.7 ± 4.1	10.9 ± 2.6	3.9 ± 2.1	6.6 ± 1.6	55.2 ± 4.0
	5	-17.1 ± 2.7	-46.0 ± 4.0	8.5 ± 2.0	8.0 ± 2.4	6.2 ± 2.1	55.0 ± 3.3
		-10.3 ± 1.9	-38.5 ± 3.7	10.3 ± 1.4	7.1 ± 3.1	7.1 ± 1.9	55.7 ± 4.2
	6	-10.6 ± 2.3	-34.5 ± 3.3	10.7 ± 1.2	5.3 ± 2.6	6.0 ± 1.9	53.9 ± 3.7
		-10.3 ± 2.0	-33.6 ± 3.9	9.9 ± 1.0	5.2 ± 3.2	6.3 ± 1.6	54.7 ± 3.5
	7	- 8.6 ± 1.8	-38.0 ± 4.0	9.1 ± 1.8	5.2 ± 2.7	6.1 ± 2.1	54.9 ± 3.7
		- 9.4 ± 2.6	-36.8 ± 3.5	13.7 ± 2.2	6.2 ± 2.5	6.4 ± 1.8	55.0 ± 3.9
Thyroxine treatment	8	- 8.4 ± 1.9	-43.6 ± 4.2	4.5 ± 0.9	4.8 ± 2.4	5.8 ± 1.3	55.2 ± 4.2
		- 8.0 ± 2.1	-31.2 ± 3.6	5.6 ± 1.2	3.4 ± 2.6	5.9 ± 1.6	55.5 ± 4.1
	9	- 4.0 ± 1.2	- 3.1 ± 1.6	4.6 ± 1.5	4.6 ± 1.8	5.1 ± 1.9	55.2 ± 4.3
		+ 2.1 ± 1.2	-12.2 ± 2.6	3.1 ± 0.8	3.2 ± 2.1	4.4 ± 1.8	55.1 ± 4.2
	10	0.0 ± 0.9	-16.7 ± 2.7	3.6 ± 1.4	2.6 ± 2.0	2.4 ± 1.9	54.8 ± 4.1
		+ 1.1 ± 0.8	+ 3.0 ± 2.6	4.6 ± 1.0	2.9 ± 2.4	4.2 ± 2.0	54.5 ± 4.3
	11	+ 2.3 ± 1.4	0.0 ± 2.1	2.4 ± 1.0	2.6 ± 2.1	4.7 ± 1.6	53.8 ± 4.0
		+ 2.8 ± 1.7	+ 2.8 ± 3.1	3.7 ± 0.9	2.7 ± 2.6	4.0 ± 1.3	53.7 ± 3.8
	12	+ 3.3 ± 0.9	+ 4.6 ± 2.9	2.5 ± 0.8	2.5 ± 2.3	2.7 ± 0.8	53.4 ± 4.2
		+ 2.5 ± 1.0	+ 2.6 ± 1.9	3.5 ± 0.7	2.2 ± 2.6	2.5 ± 1.2	52.9 ± 3.7
	13	+ 4.1 ± 1.3	+ 6.6 ± 2.6	2.7 ± 0.5	2.6 ± 2.5	2.8 ± 1.1	52.5 ± 2.4
		+ 3.2 ± 1.2	+ 6.0 ± 2.6	4.4 ± 0.8	2.5 ± 1.3	2.4 ± 0.8	51.7 ± 3.9
	14	+ 3.6 ± 1.1	+ 5.7 ± 3.1	3.2 ± 1.0	2.5 ± 2.4	3.2 ± 1.2	50.8 ± 2.8
		+ 3.8 ± 0.9	+ 6.2 ± 2.9	4.0 ± 0.8	2.7 ± 3.1	2.3 ± 0.9	49.8 ± 3.0
Post Thyroxine treatment	15	+ 3.5 ± 1.2	+ 4.6 ± 3.1	4.0 ± 1.0	3.1 ± 2.7	3.2 ± 1.0	———
		+ 3.6 ± 1.0	+ 5.1 ± 2.8	4.0 ± 1.2	3.0 ± 2.4	2.1 ± 1.1	49.0 ± 3.6
	16	+ 4.1 ± 0.8	+ 3.1 ± 2.7	5.7 ± 2.0	5.2 ± 1.9	2.9 ± 0.8	———
		+ 1.2 ± 1.1	+ 2.4 ± 3.0	4.9 ± 1.8	4.1 ± 2.4	2.7 ± 1.2	48.5 ± 4.2
	17	- 2.0 ± 1.5	- 1.7 ± 3.4	6.7 ± 1.7	5.4 ± 2.9	2.5 ± 1.4	———
		- 2.6 ± 1.6	- 6.7 ± 2.6	6.6 ± 1.9	5.7 ± 2.8	3.1 ± 1.9	48.2 ± 3.7
	18	- 5.3 ± 2.6	- 5.9 ± 2.6	7.4 ± 2.1	7.1 ± 2.7	3.2 ± 2.2	———
		- 5.7 ± 2.0	-10.1 ± 3.4	7.0 ± 1.3	6.3 ± 2.5	4.7 ± 1.7	47.8 ± 4.1
	//						
	20	- 7.6 ± 1.9	-24.3 ± 3.6	9.7 ± 2.1	6.7 ± 2.7	5.6 ± 2.0	———
		- 7.8 ± 2.2	-20.1 ± 4.2	10.1 ± 1.8	6.5 ± 1.9	6.2 ± 3.3	47.6 ± 4.2

Each value given is the mean ± S.E. of 8 animals, and are for each 12 hour period during the time of the experiment: days 1 - 20. Sodium and chloride values are expressed as μ equivalents (12 hour period.) For the ion values under bathing fluid '+' represents uptake through the skin from the fluid, and '-' loss to the fluid.

completion of the seven days treatment with thyroxine, the values slowly dropped, until a state of equilibrium as regards this ion had been reached.

Thyroxine treatment, at the level administered in these experiments, brought about a net gain of chloride after approximately four days treatment, values of net gain being 13 μEq. per twelve hours. Two days after thyroxine treatment had ended, however, the values fell gradually until by the sixth day after treatment net losses of the same order as those of the first control period were found.

As a check against increase in bathing fluid concentration caused by evaporation, three plastic chambers were set up with no animals in them. They contained 100 ml. of tap water and were gently aerated for twelve hours in the experimental room. The volume decrease was 0.9 ml. and, using the analytical methods described, the subsequent change in concentration was insignificant.

2. Effect of thyroxine on urine sodium and chloride content.

The twelve hour losses of sodium and chloride in the urine are given in Table 12. The losses of sodium can be seen to be variable, but nevertheless vary between certain limits, namely 16.3 and 8.0 μEq. per twelve hour period, these two values being the upper and lower limits of variance respectively. The chloride loss also shows variance but of a slightly lower order, the upper and lower limits recorded being 10.5 and 2.6 μEq. per twelve hours, the average value over all the periods being 5.63 μEq. per twelve hours. After four days of thyroxine treatment the sodium loss

values dropped to between two and four μEq. per twelve hours, whilst a similar pattern was seen with the chloride values, the losses being of the order of 2.5 μEq. per twelve hours. A decrease in the excretion of these two ions is, therefore, obvious and after thyroxine treatment the values rose again to their former levels.

As well as a decrease in the total amount of ions excreted, a decrease in concentration occurred. The total concentration of ions in the urine before thyroxine treatment was 2.48 μEq./ml. and after four days thyroxine treatment became 1.64 μEq./ml. Both ions showed a decrease in concentration, but sodium was the most affected, dropping from 1.59 μEq./ml. before to 0.88 μEq./ml. after treatment, thereby showing a fall from 64% of the total ionic excretion to 53% of the total.

3. Effect of thyroxine on urine volume.

The urine volume per twelve hour period was consistent, especially over the last eight periods before treatment with the hormone, but after four days thyroxine treatment the value decreased to approximately half its normal value. There appears, therefore, to be an increased resorption of water either in the kidney tubule or from the urinary bladder. After thyroxine treatment the urine volume slowly increased to normal values again, during the six day period for which it was investigated.

4. Effect of thyroxine on the net sodium and chloride balance.

The total loss of sodium, that is, injected minus the amount lost via the skin and urine was approximately 13 μEq. per twelve hours during the control periods, but four days after the beginning

of thyroxine treatment a total gain was observed of the order of 7 uEq. per twelve hours. A similar relationship is seen when chloride is considered, a total loss of 30 uEq. per twelve hours was recorded before thyroxine treatment and after four days of treatment with 25 ugm. thyroxine per animal per day values of 10 uEq. per twelve hour period were found, this latter figure being a total gain of chloride. Thyroxine therefore causes an alteration in the ion balance of the toad Bufo bufo, as regards sodium and chloride movements. This effect is brought about partly by the action on the absorption of sodium and chloride through the skin and partly by the action on the urine water and ions.

5. Effect of thyroxine on the body weight of animals.

Body weights remained constant during the control period of seven days. With thyroxine treatment, however, weights gradually decreased (see Table 12). Weight loss occurred despite a net gain of ions which was presumably accompanied by both an increase in water uptake and a decrease in urine output and both of these processes normally result in an increase of body weight. The animals were starved during the whole of the experiment and for twenty one days prior to the control period and, as the results from the latter period show, a more or less steady state has been acheived. After the thyroxine period, analysis was continued and the rate of loss of weight was reduced as daily values became much closer together. The weight loss values tend to be slightly masked by the standard errors, but these arise solely as a result of expressing the weight as a mean of eight animals. The variance,

therefore, is purely a statistical one, as can be seen from the fact that the standard error has roughly the same value at each period.

D. DISCUSSION.

Thyroxine injection (25 ugm./toad/day) increases the amount of ions taken in through the skin of Bufo bufo but, although only sodium and chloride were measured, a possible effect on other ion species cannot be ruled out. In addition, thyroxine appears to cause an increased resorption of ions and water from the bladder, or possible the kidney. It has been shown that thyroxine increases the active, inward transport of sodium through the isolated toad skin and bladder (see III,C) and also increases the passive permeability of the bladder to water (see II,C). In addition to this work, both Embden and Adler (1922) and Saito (193[illegible] have shown that the skin of thyroxine fed frogs and skin treated *in vitro* with thyroxine show an increased permeability to dyestuffs and water. From these *in vitro* studies it is possible to infer that similar changes may occur in the intact animal.

Although there are some reports of levels of hormonal iodine in Amphibia (see Leloup and Fontaine, 1960), there are no direct measurements of the titre of thyroid hormones in Amphibian plasma. One report by Genest and Adams (1957) exists where the level of thyroxine was estimated by evaluating "the minimal amount of thyro-

xine necessary to induce moulting and to prevent the usual cessation of moulting and maintain a normal skin in the thyroidectomized newt". This method is rather unsatisfactory for the method of replacement thereby gives very high figures and the needs for thyroxine are probably highest during moulting. The dose of 25 ugm. per 50 gm. toad per day used in these experiments may be considered as a reasonable dose level, although they are of the order of figures obtained from mammalian work (Pitt-Rivers and Tata, 1959, pp. 41-43).

The net loss of ions through the skin into the bathing fluid is not surprising, since it has previously been shown in Bufo bufo, as in other Amphibia, an almost steady state is maintained with a tendency to lose ions gradually when immersed in tap water (Krogh, 1939). In addition, Jørgensen, Levi and Ussing (1949) showed that the mere pricking of the skin of Amphibia produced a salt loss lasting for several hours. Although the 'pricking' in these experiments was kept to the minimum, this effect undoubtedly could contribute to the net salt loss. Cutaneous loss can also be expected since the animals received an injection of saline each day. Jørgensen (1954) has shown that such injections enhance the cutaneous loss of electrolytes in order to maintain ionic equilibrium within the body, although under normal conditions the loss through the skin is comparatively unimportant, the sodium and chloride being excreted mainly through the kidneys.

Thyroxine treatment decreased the amounts lost through the skin, until a net gain was observed. This observation may be re-

lated to *in vitro* studies where an increased active transport of sodium was found when thyroxine was added to the serosal surface of the skin (III,C). This increase would explain the results obtained and also explain how the net loss of chloride would become a net gain, for the increased sodium movement would create a larger driving force across the skin which would tend to pull more chloride ions into the animal. A similar comparison can be made between results obtained *in vivo* and *in vitro* on the bladder. The results of the present experiment show that the urine concentration, as well as urine volume, decreases after thyroxine treatment. Toad urine is normally hypotonic to the blood (see Leaf, Anderson and Page, 1958) and the present *in vivo* studies show that thyroxine leads to increased resorption of water, as seen by the decrease in urine volume, and also increased resorption of s sodium and chloride, for the concentration of each ion per milliliter of urine decreases. When the mucosal fluid is made hypotonic to the serosal fluid an increase in water flow to the serosal surface is seen after the addition of thyroxine to this side of the bladder (see II,C). In addition, thyroxine on the serosal side of the bladder bathed on each side by the same medium, results in an acceleration of active sodium transport across the bladder (III,C). This is not to say that stimulation of sodium transport does not occur in the presence of an osmotic gradient, but the demonstration of such 'downhill' transport is technically difficult.

The results obtained, as depicted in Table 12, indicate that

after thyroxine treatment had ended and injection of saline alone resumed, the animals showed a gradual return to the control levels previously observed, with the exception of the weight loss. This indicates that the effects seen during hormonal treatment were due to the hormone alone and not due to any deterioration in the condition of the animals.

The more exact measurement of the action of thyroxine on different parameters of salt and water balance of the toad in these experiments is supported by the cursory observations of some other workers. Koefoed-Johnsen and Ussing (1949), for example, found that injection of thyroxine into the axolotl caused a net uptake of sodium and chloride in each case and Levinsky and Sawyer (1952) noted an increase in the weight of cloaca ligatured frogs after thyroxine treatment.

The resemblance between the ion balance during naturally occurring moulting and the thyroxine induced effects observed in the experiments is marked. For, just as thyroxine has been shown to affect the actual physical process of moulting (Jørgensen and Larsen, 1961), so it appears from the present results that thyroxine controls the physiological processes involved in moulting. Jørgensen and Larsen (1961) have shown that thyroxine induces moulting in Bufo species and this is a time when sodium uptake, during 'natural' moulting, is increased (Jørgensen, 1949). The results obtained after thyroxine injection indicate that this hormone affects ion balance and the possibility exists that, in the natural course of events, thyroxine may influence the moulting

process and ion changes concurrently. That the thyroxine effect on ion balance is independent of shedding of the skin is shown in the present experiments in which non-moulting animals responded to hormonal treatment.

The work of Hickman (1959) on the flounder has shown that concurrent changes occur in thyroid activity and osmoregulation when the animal is transferred to different media. The results imply that when the animal is subjected to a more concentrated medium, a condition where salt excretion is at a premium, there is an increase in thyroid activity, together with increased metabolism. The conclusion reached is that the thyroid hormones control ionic regulation and metabolism, at least in this species of fish and although these results were obtained in a different group of animals the correlation between ion regulation and thyroid activity in both these and the present results is strong.

The neurophypophyseal hormones have been shown to alter both the cutaneous salt and water balance (see Sawyer, 1956) and also to increase water and ion resorption from the bladder (Ewer, 1952a). Both these effects were confirmed <u>in vitro</u> by Furhman and Ussing (1951) on skin, and Bentley (1958) on bladder. The neurohypophyseal hormones have, however, been shown to affect the rate of glomerular filtration, as well as water resorption from the distal tubule and water resorption from the bladder (see Sawyer, 1956). The effects observed even after minute doses of the pressor extracts are large compared to those seen after thyroxine injection in the present experiments. Such an effect of thyroxine on the

glomerular filtration rate has not been observed in Amphibia, but results from mammals indicate that the hormone increases the filtration rate and in addition, increases the clearance rates of various substances (Corcoran and Page, 1947), the latter implying an increase in permeability processes. The same authors show that in myxoedema there is a reduction in tubular resorptive capacity of the kidney. This clinical report is supported by work on rats by Stephan and his co-workers (1959a,b; 1960; 1961), where they found that inhibition of thyroid secretion led to an increase in sodium and chloride output in the urine, but injection of thyroxine caused a decrease in such excessive ionic output in restoring the balance to normal. The kidney tubule of the rat has been shown to resorb sodium actively from the tubule into the plasma (Windhager and Giebisch, 1961) and it is possible to draw a relationship between the kidney tubule and the toad bladder, for both perform similar procedures, although differing anatomically (Leaf, 1960b). If the analogy is correct then, since thyroxine affects ionic transfer across the bladder, it is equally possible that it may exert some control over tubular resorption. Obviously the effect would not be as great as that exerted by vasopressin, but nevertheless, as is shown in the present experiments, thyroxine does alter both the urine volume and the urine concentration of ions in the toad. Thus it may be that the thyroid hormones control, to a certain extent, both the kidney tubular resorption of ions and ionic resorption from the bladder, although in the present series of experiments it is impossible to distinguish the separate

effects.

Other hormones have been shown to alter the salt balance of adult Amphibia, in particular the secretions of the adrenal cortex. Koefoed-Johnsen and Ussing (1949) found that injection of ox ACTH induced a pronounced increase in total and net uptake of sodium through the skin of the axolotl. It has also been shown that saline-loaded adrenalectomised frogs excrete more sodium via the kidney than do normal frogs under similar circumstances (Fowler, 1957), whilst there is increased loss of sodium through the skin due to the increased permeability (Jørgensen, 1947) and at the same time the active transport of sodium inwards is decreased. Sawyer, Travis and Levinsky (1950) were, however, unable to detect any effect on water balance in frogs after injection of adrenocortical extract, cortisone, desoxycorticosterone acetate or ACTH. The evidence is therefore a little confusing, but it is possible to say that the adrenal steroids may be essential in preventing excessive dehydration in Amphibia. Schoeffeniels and Baillien (1960) have, in addition, demonstrated that the sex hormones increase the sodium shift across the frog skin and thus it appears, both from *in vivo* and *in vitro* work, that several hormones exert effects of differing magnitudes on the permeability of the skin.

Previous observations do not appear to have been made on the short term effect of thyroxine injection on the body weight of Amphibia, although Warren (1940), using frogs, found that by feeding thyroxine twice weekly a decrease of 7.8 gms. in ten weeks

was induced, whilst the control animals lost only 2.9 gms. in twelve weeks. In the present experiments thyroxine injections caused a loss in weight within the first week of administration, thus it appears, by determining weight loss, that thyroxine has an effect on the metabolism of the adult Amphibian. There also seems to be a latent period of action on this parameter, for the weight loss does not begin to fall to any extent until the third day after thyroxine injection, this response resembles the effects seen on the basal metabolic rate in mammals (Barker, 1956).

It is by no means clear that there is an effect of thyroxine on the basal metabolism of adult Amphibia, as measured by oxygen consumption. For, although Gayda (1922) and Galton and Ingbar (1962a) found no effect of thyroxine on the respiration rate of toads and frogs, respectively, the work of Donoso and Trivelloni (1958) supports that of Warren, in that injection of either thyroxine or triiodothyronine produced an increase in oxygen uptake of toads within a few days of administration. Weight-loss, however, can be interpreted as an increased mobilisation and utilisation of available reserves within the body, as has been seen in human hyperthyroid patients and which has also been seen in Amphibia and other lower vertebrates as a result of thyroid administration (see Dodd and Matty, 1963). Such a weight-loss in the present experiments supports the concept that thyroxine may have an effect on the metabolism of adult Amphibia in a manner similar to that already shown in other groups. Leloup and Fontaine (1960), after reviewing the literature concerned with metabolism of lower verte-

brates as affected by thyroxine, conclude that the thyroid in the lower vertebrates, contrary to classic beliefs, may be implicated in respiratory exchanges.

It may be of significance that the changes in ion balance occur slightly before any detectable change in metabolism, as measured by weight loss, thereby implying that the hormone injections produce their effects on ion balance before any effect on metabolism, the permeability processes being the primary effects of thyroxine. Thyroxine, therefore, affects the permeability both of the skin and bladder membranes in vitro, concurrent with alterations in metabolism, and it is extremely likely, on the basis of the in vitro work, that the two processes are intimately connected.

SECTION VI

GENERAL DISCUSSION

The spectrum of structure and function affected by the thyroid hormones is wider by far than that of any other hormone and they have attracted much attention for this reason. Attention has been focused on the peripheral effects of the thyroid hormones and in general it has been found that the physiological role of the hormones is in the maintenance of cellular activity. For, as Barker (1962) has pointed out, the elevation of the metabolic rate by several hundred percent, loss of weight and excess mobilisation of body tissues by thyroid hormones, is a stress reaction and the function of the thyroid more properly belongs at ordinary levels of regulation of cellular function.

Dodd and Matty (1963) have shown that in the lower vertebrates, the thyroid exerts an effect on many processes and the same is true in mammals (Pitt-Rivers and Tata, 1959). Despite the diversity of processes known to be affected, much attention has been focused on a single basis of action, such as that on the fundamental level of cellular metabolism. Much work along these lines has been performed, especially on specific enzymes or enzyme systems, but the evidence is conflicting and inconclusive (see Pitt-Rivers and Tata, 1959). It was soon realised, however, following work in other fields, that structural integrity of the cell or cell organelles was an essential factor in understanding the action of any controlling factor. Disruption of cells and

their contents disturbs the spacial relationships within the cell and thus interferes with the normal processes of regulation of cellular activity. After this realisation many workers performed experiments on whole organelles in an attempt to localise the point at which thyroxine exerted its controlling influence. Tapley (1956) and Lehninger (1956) investigated the effects of thyroxine on intact mitochondria and obtained a distinct swelling, which was reversible upon the addition of adenosine triphosphate. Correlated with this swelling was an alteration, at higher doses of thyroxine, in the ratio of the rate of exchange of inorganic phosphorus and oxygen uptake. It was postulated that thyroxine exerted its effect by altering the permeability of the mitochondria to ions, water and substrates and in this way controlled other cellular processes which are dependent upon these primary processes. Tapley and Hatfield (1962), reviewing the literature on these mitochondrial effects, suggest that many of the effects of thyroxine both <u>in vitro</u> and <u>in vivo</u> may be brought about by an action of thyroxine on the mitochondrial membrane. Such changes, which alter the rate of entry and exit of substances, must of necessity result in profound alterations in the economy of the cell.

Recently, Tata (1962) has demonstrated that thyroxine, when used in physiological doses, in the first stages of its action in the rat, preferentially affects processes or enzymes which require some form of structural integrity. The overall pattern of changes, which occur as a result of thyroid hormone administration, in a variety of cellular activities resembles in many ways the

changes observed during tissue and body growth. The work shows that amino acid incorporation precedes any alteration in enzyme activity and this illustrates that the hormones may be intimately concerned with the interplay between cellular structure and activity. Hechter (1955) has drawn attention to the possible role of endocrine secretions in the regulation of this relationship and much recent work has supported this concept.

In the Amphibia, emphasis has been placed upon the role of the thyroid hormones in metamorphosis, but there is a diverse spread of effects throughout this group, such as is seen in mammals (Pitt-Rivers and Tata, 1959). Few attempts have been made to investigate thyroid effects on permeability in any group, although the several clinical reports of effects in mammals (de Gennes and Bricaire, 1951) and reports available from fish work (Fontaine, 1956) show that in both groups the thyroid helps in the elimination of water and retention of chloride. Some experimental work has been performed on mammals (Pitt-Rivers and Tata, 1959), but this has mainly been concerned with the diuretic effect of the hormones in hyperthyroidism. Recently, however, Stephan and his co-workers (1959a,b; 1961) have shown that hypothyroid rats show decreased resorption of ions from the kidney tubules and that thyroxine injection restores the ion balance to normal levels. The implication from this work is that thyroxine maintains the active ionic resorption processes, which are known to occur in the kidney tubules (Giebisch, 1962).

No <u>in vitro</u> studies have been performed on the effect of

thyroidal hormones on permeability of any tissues or membranes, except for a few reports of thyroxine effects on permeability in Amphibia. Embden and Adler (1922), for instance, observed that the application of thyroid extract to frog skin increased the water permeability of the membrane, whilst Saito (1930) found an increase in permeability to dye-stuffs, measured *in vitro*, after feeding frogs with thyroid glands. Asher (1923) noticed that thyroid extract also caused an increase in the penetration rate of methylene blue into the nictitating membrane of frogs. The studies of Gellhorn (1929) and Gellhorn and Northrup (1933) showed that thyroxine, at very low dose levels, caused an increase in glucose absorption across the perfused gut of *Rana esculenta*, the effect being seen after ten minutes of application of the hormone and becoming more pronounced after twenty minutes incubation. Studies since this time on intestinal movement of glucose in frog (Csaky and Fernald, 1961) and mammals (Crane, 1962) have indicated that the transfer process is an active, energy-requiring process located in the mucosal epithelial cells. The experiments of Gellhorn and Northrup, therefore, illustrate that thyroxine acts upon an active transport system in Amphibia. This was the first demonstration of such an effect in any vertebrate.

The present study has shown that both thyroxine and triiodothyronine exert a variety of effects on at least two Amphibian tissues - the skin and bladder of the toad. The doses of hormone required to elicit changes in the parameters of activity measured, were of the order of those present in mammalian plasma. Measure-

ments of the titre of thyroid hormones in Amphian plasma have not been made and only indirect determinations exist (see Leloup and Fontaine, 1960). Although the majority of studies were persued using a concentration of 10^{-6}M for both hormones, this gave only a quantitative difference from the increases obtained at 10^{-8} or 10^{-9}M. It is interesting to note that Frienkel, Ingbar and Dowling (1953) found that 40% of a 200 ug. % solution of thyroxine was adsorbed onto glassware and that the percentage adsorption decreased with increasing concentration. At the levels used in the present experiments it is likely that the concentration that the tissue was exposed to was, indeed, appreciably lower than that initially present, due to this adsorption factor, thus presenting an even more physiological dose to the tissue.

Water permeability is increased in the presence of an osmotic gradient and this passive process is a result of the chemical and physical gradients which exist across the membrane. The hormones appear to cause an alteration in the fine structure of the membrane, thus allowing a freer flow of water down the existing osmotic gradient. In the absence of an artificial osmotic gradient the force causing water transfer across the bladder wall is purely an osmotic one created by the potential which is developed across the membrane. The transfer of ions creates a slight osmotic pressure which results in a small water movement. The ionic movement is stimulated by the hormone and this in turn causes a slightly greater movement of water. It appears from experiments performed both in the presence and absence of an osmotic gradient,

that thyroxine increases the area available for diffusion of wat in addition to increasing the active, energy-dependent process of active sodium transport. The former effect allows a freer diffusion of non-transported molecules and ions and offers an explanation of the lack of increase in potential after thyroxine addition.

The potential measured across any membrane relies upon the total quantities of ions present in the bathing fluids on each side of the membrane, but the short-circuit current is a measure only of active ion movement. Sodium is actively transported in the toad skin and bladder, and since it is stimulated by thyroxine an increase in potential would be expected. This, however, does not occur and measurements of anion fluxes show that maintenance of the almost constant potential can be explained as a result of increased movement of anions, particularly chloride ions. This anion shift tends to equalise the concentration of ions on each side of the membrane despite the increased sodium transport. Thyroxine and triiodothyronine not only cause a more ready diffusion of anions, cations (for passive sodium efflux across the membrane is increased) and water, but also stimulate oxygen consumption as a result of the effect on enzymic processes within the cell.

The present work on the increase in respiration of tissues <u>in vitro</u> after thyroid hormone addition stands in contrast to much mammalian work where negative results have been obtained.

During recent years, however, evidence has been presented by several workers that *in vitro* increases in tissue oxygen uptake are seen after the addition of thyroid hormones to the bathing medium (Gross and Pitt-Rivers, 1953; Thibault and Pitt-Rivers, 1955; Yoshimiro, 1956; Jacob, 1962). Although the evidence for any *in vitro* metabolic effect in any group of vertebrates is a little confusing, the work reported in this study shows that both thyroxine and triiodothyronine cause an increase in metabolism after addition to the toad skin and bladder *in vitro*. This confirms several reports of metabolic effects of thyroid hormones in lower vertebrates (see Leloup and Fontaine, 1960), and the evidence indicates that the thyroid may be implicated in metabolism in these groups, as it is in mammals. In the latter goup, however, the more well-known effects of increase in oxygen uptake of whole animals after thyroid treatment, has in most cases been achieved with high doses of thyroxine, and further has been associated with loss of weight. The recent studies of Tata (1962) and Tata, Ernster and Lindberg (1962) have suitably demonstrated that administration of the non-toxic dose of 10 micrograms of thyroxine per 100 grams body weight to thyroidectomised rats every fourth day raised the metabolic rate. In this case, however, a growth response, rather than a catabolic one was observed in the treated animals.

The relationship of oxygen uptake to sodium transport has been investigated in several tissues, such as frog skin (Zerahn, 1956; Leaf and Renshaw, 1957), toad bladder (Leaf, Page and Anderson, 1959), gastric mucosa (Davenport and Chavre, 1952) and

kidney tubules (Lassen and Taysen, 1961). The ratio of sodium transported to oxygen consumed has been shown, in all these tissues, to be of the order of sixteen or eighteen to one. Such a ratio has been confirmed in the bladder and skin of the toad, but it is found that forty minutes after the addition of thyroxine the ratio has fallen to eight to one. Analysis of this fall in the ratio has shown that the oxygen consumption is increasing at a greater rate than that required by the sodium transport mechanism, assuming a sixteen to one ($Na:O_2$) relationship is maintained under the stimulated conditions. It is evident from the data that thyroxine is not only stimulating that part of the metabolism directed towards sodium transport, but, in addition, is increasing general cellular metabolism. In the work reported here the increase in metabolism appears to be secondary to an initial effect on the cell membrane, which influences the exchange of substances across this membrane. This further regulates availability of enzymic substrates and hence exerts a control of metabolism. Anderson (unpublished work, reported by Ussing, 1959) has demonstrated that following ad adjustment in the potential across frog skin there is a period of 10 to 15 minutes before the oxygen consumption adjusts to the new load. Thus, there appears to be a store of energy which can be drawn upon when the demand from the active ion transport increases. The increases in sodium transport and oxygen consumption are more or less concurrent in the present experiments and thus it is unlikely that thyroxine is simply stimulating the movements of ions across cell membranes, but is in addition, either

penetrating the cell and affecting enzyme systems, or controlling the influx and efflux of cellular substrates through a fundamental control of cellular permeability. In this way it brings about the more manifold effects on permeability.

The relationship between water movement and oxygen consumption in sodium and non-sodium media and in bladders immersed in these media in the presence of thyroxine is depicted in Fig. 22. Oxygen values are plotted from Table 10, the mean of the difference of oxygen uptakes in choline medium plus thyroxine being added to the control values of column 1. Similarly the mean of the difference of oxygen uptake in sodium medium plus thyroxine is added to the control values from Table 10, column 2. The difference between oxygen consumption in sodium and choline media is that part of the total respiration used for sodium transport (Zerahn, 1956). A comparison of the water loss values indicates that in the absence of sodium there is a slight decrease in the permeability of the membrane. The cross-hatched areas indicate sodium-involved enzyme systems and are only attempts at quantitative representation, R_1 and R_3 being the only values which can be directly determined. It can easily be seen that even in sodium-free medium there is an increase in metabolism, which can only be a result of stimulation by thyroxine of those enzymes not concerned with sodium transport. It is of interest to note that Bruhn (1941) found a correlation between the water exchange and increase in metabolism of the dog after oral thyroid administration. This observation may be related to those of the present study, although the dose of 2 gm.

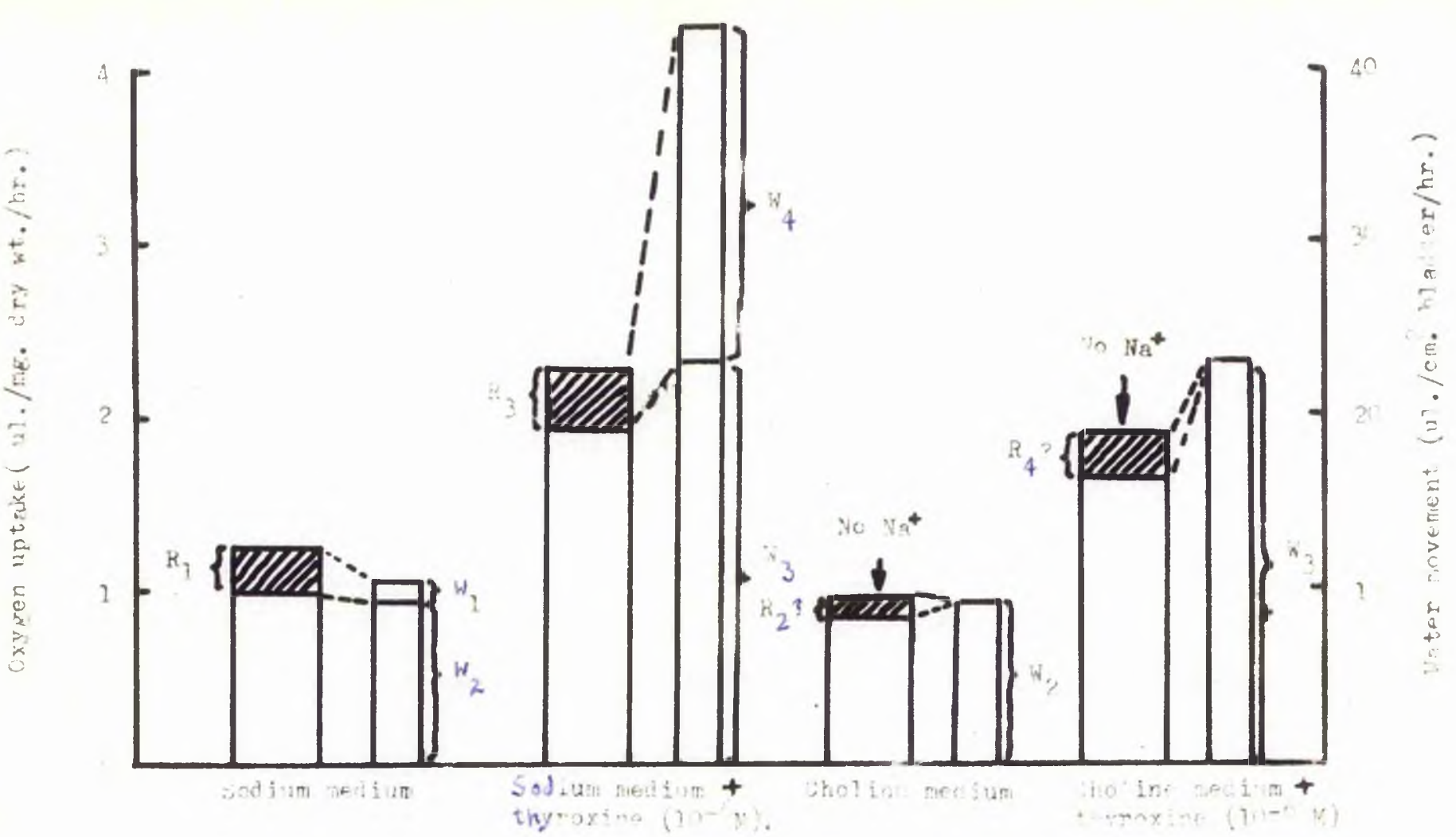


Fig. 22. Schematic diagram to illustrate the possible action of thyroxine on enzyme systems and correlation between oxygen uptake and water movement. Oxygen consumption : wide column ; water movement : narrow column. Cross hatched areas (R_1-R_4): enzyme activity resulting in sodium transport. W_1 : difference between water movement in sodium and choline media. W_2 : water movement in absence of sodium. W_3: water movement in absence of sodium but in presence of thyroxine. W_4 : water movement associated with presence of sodium. For further explanation see text.

per day for 10 days used by Bruhn was rather high.

It was demonstrated by Gross and Pitt-Rivers (1953) and Barker (1955; 1956) that the activity of triiodothyronine as measured on oxygen concumption of tissue slices from previously injected rats, was four or more times greater than thyroxine. Tata (1962) has, however, pointed out that if one plots time against percent change in the metabolic rate in mammals, following a single equimolar injection of thyroxine or triiodothyronine, the areas occupied by the two curves will be almost equal. Barker (1956) also investigated the effects of certain analogues on tissue respiration in mammals and found that TETRAC was about three-quarters as active as thyroxine, whilst TRIAC was about a quarter more active than thyroxine. As assayed on the tadpole growth response, triiodothyronine again shows a four fold increase above that of thyroxine (Roche, Michel, Truchot and Wolf, 1955), whilst the proprionic analogues are a hundred and thirty times as active as thyroxine (Bruice, Winzler and Kharasch, 1954). Donoso and Trivelloni (1958), working on various species of adult toads, found that although triiodothyronine had a shorter latent period (four days) than thyroxine (seven days) following equimolar injections of the hormones, they both caused the same <u>total</u> response. The present study, investigating the comparison between the hormones, has shown once more that triiodothyronine has a shorter latent period of action than thyroxine, which is presumably caused by the faster rate of entry of the former substance into the cells (Roche and Michel, 1960). In addition, the results show that

thyroxine, at least on the tissues used in this study, causes both a greater maximal and a greater total effect than triiodothyronine on all the parameters of activity measured. Thyroxine produced a response almost twice that of triiodothyronine, as measured by the total area under the curve. None of the analogues showed any effect on sodium transport or metabolism, but TETRAC and TRIAC were found to have a fleeting effect on water permeability across the bladder. Why these two substances caused this water loss response is an unsolved problem, especially in view of their lack of effect on other cellular processes.

The evidence obtained from the present studies points to the fact that thyroxine and triiodothyronine are the peripherally active forms of the hormone. It is interesting to note that Galton and Ingbar (1962a) found that in the adult frog, as compared to the tadpole, there are no enzymes capable of causing metabolism of thyroxine or triiodothyronine to the acetic acid analogues. This is of interest for, in the present work, no effect of the analogues has been seen except that of TETRAC and TRIAC on water loss, although the parent hormones showed a variety of effects. Thus it appears unlikely, but not impossible, that thyroxine and triiodothyronine when applied <u>in vitro</u> to the tissues used, are not metabolised to the analogues, although they must undergo some breakdown. Tata (1962) has pointed out, however, that it is not known whether the thyroid hormones can act at their sites of action only in their free forms or whether their metabolism precedes or follows their action.

The results of incubating bladders under isosmotic condition with thyroxine bathing either surface, have indicated that the ra of penetration of the hormone is four times faster through the serosal surface than through the mucosal. Both water movement studies, where thyroxine results in a lower response when on the mucosal surface and short-circuit current measurements, where thyroxine on the mucosal surface has an effect a quarter of that when on the serosal surface both in time of response and maxima produced support this concept. The effects of the hormones on the ionic content of the cells also supports this for, when applied to the serosal surface, thyroxine results in an immediate alteration of the ionic (Na^+ and K^+) content of the cells, whereas when on the mucosal surface the effect is much slower.

From a consideration of the effects of thyroxine on the Koefoed-Johnsen-Ussing model for the development of potential, it can be seen that thyroxine causes an increase in the passive permeability of the mucosal surface and also stimulates the forced exchange of sodium and potassium across the serosal surface. When applied to either side of the membrane, the hormone causes differences in ionic exchange due to its effects on the side of the membrane to which it is applied. On the mucosal surface it creates an increased passive diffusion of sodium into the cell, thereby providing more sodium for the 'pump' which, in turn, responds by increasing slightly in activity. When on the serosal side thyroxine stimulates the 'pump' directly, after altering the permeability of the serosal membrane. The increased activity of the 'pump'

thus creates a larger gradient which pulls sodium into the cell across the mucosal surface. A limit would thus be set by the diffusion rate across the mucosal surface, but due to its faster rate of penetration through the serosal membrane it is postulated that the hormone also affects the mucosal barrier. This enables more sodium to enter the cell and results in a faster flow of sodium across the total membrane.

The results from studies of thyroxine action on the ionic content of cells indicates that the hormone affects cellular permeability to the different ions before affecting metabolism or sodium transport. There is evidence, therefore, to indicate that the primary effect of the hormone is on the cell membrane and that this effect is followed by a further, more profound effect, after the hormone has penetrated into the cell. This further penetration leads to a more general alteration in permeability, due to the effects on the enzymic mechanisms which control the sodium 'pump' and also exert a control of 'pore' size. The effect of vasopressin has also been analysed in terms of the Ussing model and it has been shown that the effect of the hormone is to control the permeability of the outer mucosal cell surface (Curran, Herrera and Flanigan, 1963; Frazier and Hammer, 1963). In this way it controls the amount of sodium which enters the cells and therefore exerts only an indirect effect upon the sodium pump. The effect appears, therefore, to be slightly different from that of thyroxine where, although the initial process may be similar to that shown by vasopressin, an enzymic effect, at least after hormonal pene-

tration, cannot be excluded.

The mechanism of active transport has been the subject of a great deal of work and it has been clearly shown that it is dependent upon either aerobic or anaerobic metabolism (Ussing, 1960c Studies of the effects of different inhibitors have been carried out with the purpose of obtaining information about the nature of the biochemical reactions underlying ion transport. Evidence has been obtained from many sources that dephosphorylation of adenosine triphosphate (ATP) is the source of energy for the sodium pump. Caldwell and Keynes (1957), for instance, succeeded in partially restoring the sodium efflux in a poisoned nerve by injection of ATP, the amount of sodium extruded being roughly proportional to the amount of ATP injected. The subject of participation of ATP in active transport has been discussed by Caldwell (1959). Interest has again been aroused in these high-energy compounds, particularly in connection with hormone effects. Orloff and Handler (1962) have shown that the increase in permeability of the toad bladder produced by cyclic adenosine monophosphate resembles in all respects that caused by vasopressin. The results are consistent with the view that vasopressin exerts its effect in toad bladder by stimulating the production of cyclic adenosine monophosphate. In addition, Anderson and Brown (1963) have shown that arginine vasopressin influences the adenyl cyclase enzyme system in the kidney cortex and medulla, with the result that the enzyme becomes more effective in catalysing the formation of adenosine monophosphate. Haynes, Sutherland and Rall (1960)

have also implicated cyclic adenylic acid in a scheme of action of adrenocorticotrophin and epinephrine, while they have also suggest that the wide distribution of cyclic adenylic acid is compatible with the idea that it serves as an intermediate in other hormonal control mechanisms which are as yet unknown. No such investigations have been performed on any membranes using thyroxine, thus the relationship of the thyroxine effects on the cyclic AMP molecule remains to be examined. That the thyroid hormones do affect enzymic processes is evident from their effect on basal metabolism and the possibility exists that the hormones may be influencing enzyme systems within the cell.

The relation of sodium transported to ATP hydrolysed has been investigated by Bonting and Caravaggio (1963) in a number of tissues and they have demonstrated the presence of a sodium-potassium-activated ATP-ase in toad bladder. The results from th tissues investigated indicate that a ratio of 2.56 equivalents of cation are transported per mole of ATP hydrolysed. These observations strengthen the assumption that the sodium-potassium-activated ATP ase is closely related to the active cation transport involved in the transport of salt and water across epithelial membranes, as well as across other cellular membranes. Several workers have isolated enzymes from different tissues which are activated by sodium and potassium and they have, for instance, been found in crab nerves and mammalian brain (Skou, 1961;1962), erythrocytes (Post, 1961) and kidney (Wheeler and Whittam, 1962). From this work it appears that these high-energy compounds may be

participating in active cation transport and it is possible that thyroxine may be bringing about the effects seen in the membranes used in this investigation by indirectly or directly acting upon these compounds.

The observation that the thyroid hormones affect the permeability of membranes brings them into the same category as a number of other hormones which have been shown to affect cellular permeability of both organic molecules and inorganic substances. Insulin, for instance, increases the rate of glucose uptake by muscle and other tissues (Randle and Morgan, 1962) as well as affecting amino acid uptake of many tissues (Wool and Manchester, 1962). In a similar manner, growth hormone enhances amino acid incorporation into protein (Brande and Knobil, 1962) although the effect of corticotrophin on such incorporation is a little confusing. For, whilst Bransome and Reddy (1963) found an increase, Kostyo and Engel (1960), although obtaining a slight increase, attributed this to contamination of the preparation with growth hormone. Eichhorn, Feinstein, Halkerston and Hechter (1961) investigated the effects of corticoids, insulin and epinephrine on amino acid incorporation into rat muscle and found that all the hormones increased the level of amino acid in both diaphragm and skeletal muscle. They suggest that these hormonal effects on amino acid accumulation may be related to alterations in electrolyte permeability.

Many hormones have been shown to influence the permeability of tissues from different animals both to electrolytes and water.

In addition to the host of work on vasopressin (Leaf, 1960a,b; 1961; Bentley, 1958; 1960; Sawyer, 1961 a,b; Heller, 1945; 1956) and adrenocorticosteroids (Crabbe 1961 a,b; McAfee and Locke, 1961 see also Holmes, Phillips and Chester Jones, 1963) other hormones have been investigated as to their effects on electrolyte balance. Such studies have involved hormones which have no connection with any electrolyte or water balance effects in the whole animal and are confined to specific target organs where they exert their major effects. Tercafs (1963) for instance, demonstrated that melanophore-stimulating hormone exerted its effect by controlling the passive influx of sodium into melanocytes, as well as increasing the frog skin potential difference without affecting water permeability. Thyrotrophic hormone, known to specifically affect the thyroid gland secretion, has been shown to increase the water content of thyroid slices (Bakke, Heidman, Lawrence and Viberg, 1957) as well as stimulating the entry of sodium (Solomon, 1961). This hormone also decreases the resistance of the thyroid follicular membrane, and reciprocally increases the conductivity during induced metamorphosis of the frog tadpole and similar changes have been noted during normal metamorphosis (Gorbman and Ueda, 1963). The catecholamines increase the permeability of the submaxillary gland of cats and dogs to a variety of non-electrolytes and the work has shown that the increase in permeability is caused by an action of the hormones on intercellular cannaliculi. The hormones appear to open up these intercellular spaces, allowing

a freer diffusion of particles through the tissue. Both histamine and seotonin have been shown to increase the permeability of the venous system of a capillary bed (Majno and Palade, 1961). The effect has been analysed as one on the intercellular spaces, with both hormones increasing these spaces and thereby allowing a freer access of substances to the basement membrane, which functions as a filter.

It is becoming increasingly clear that many hormones with entirely different biological manifestations, such as insulin, androgens, growth hormone, corticosteroids as well as the 'local' hormones listed above can profoundly affect the permeability of cells. Hechter (1955) and Hechter and Lester (1960) have drawn our attention to the possibility that hormones of diverse structure may act by controlling processes occurring at the cell surface membrane. As these authors have pointed out, however, although clear evidence is available of such action at the cell membrane it appears necessary to link changes in permeability to the stimulation of certain intracellular enzyme systems.

Maffly and Edelman (1963), after investigating the effects of a variety of inhibitors and substrates on sodium transport across the toad bladder, came to the conclusion that the coupling of metabolism to the transport mechanism is achieved by a highly organized molecular arrangement. The metabolic apparatus is therefore either in close proximity to, or constitutes a part of, the transport mechanism. The transport system, therefore, relies upon metabolic energy partly isolated from the general metabolic

pool and experimental evidence was obtained that this system is dependent upon a supply of ATP. The interesting report of Tonoue (1962) has shown that thyroxine effects may be mediated via ATP. The effect of thyroxine on oxygen consumption rate of rat-liver homogenate is dependent upon the presence of ATP in the incubating system. Some of the findings indicate that the effect is not caused by an effect on the ATP-ase activity of the homogenate. Thus the possibility exists that thyroxine, known to affect enzymes in the tissues used here (as measured by increased metabolism), may be exerting its effect in a manner related to that described by Tonoue.

Lehninger (1962) and Mitchell (1961) have pointed out that enzymes are found which constitute part of the endoplasmic reticular membrane of cells. Ernster, Siefevitz and Palade (1962) have also shown that enzymes are not only tightly bound to both cellular membranes and the membranes constituting intracellular organelles, but that the enzymes are in fact part of the membrane. The enzymic activity is altered according to the structural state of the particular membrane. Mitchell (1961) has also demonstrated that ion transport across cell membranes need not require any specialised transfer mechanism, but may be a result of a spacial anisotropic arrangement of enzymes with respect to the membrane. The concept that spatial relationships within the cell may play a key role in the coupling of metabolism and transport has also been suggested by Green (1957). This work illustrates that the arrangement of these enzymes within the membrane can lead to the

separation of Na and K, as well as other ionic separations, and this indicates that membranes contain ion-selecting mechanisms. This concept has been applied by Lehninger to hormonal action at the membrane level. He showed that respiratory enzymes not only release respiratory energy but are also capable of providing mechanical energy. That is, the solid state respiratory enzyme assembly is capable of altering its configuration, as a result of increased activity and thus, as they constitute part of the membrane, result in alterations in membrane structure. Another modality of energy transformation has been outlined, that of respiration dependent ion transfer across membranes. Respiratory energy, therefore, appears to be converted into phosphate bound energy, mechanical energy and transport energy.

The relation between biological structure and function appears to be inherent in the maintenance of normal cellular activities and any substance which alters such relationships will profoundly alter cellular activity. Such changes can easily be seen to cause alterations in permeability and the possibility exists that thyroxine, due to its known action on enzyme systems, may well be altering such enzymes which constitute part of the membrane. Tata, (1961) and Tata, Ernster and Lindberg (1962) have indicated from their work that thyroxine appears to exert some control over the relationships outlined above. The present studies extend those of Tata and again suggest that thyroxine is controlling <u>cellular</u> membrane permeability as well as the permeability of the membranes, i.e. bladder and skin, as a whole. It is postulated from the

present results that thyroxine and triiodothyronine initially affect the cell surface membrane and this potentiates transfer of materials into and out of the cell. This process in turn increases enzymic activity, directly measured as oxygen consumption and this effect further produces a more manifold effect on total membrane permeability. It is thus apparent that thyroxine, although affecting enzyme systems, may only be controlling these processes secondarily to an effect on the cell membrane.

Relationships have been drawn between the processes of ion transport across the toad bladder and the kidney tubules (Leaf, 1960a). Work performed in recent years has been reviewed by Ullrich and Marsh (1963) and the multitude or results show that the ionic processes occurring across a kidney tubule can be directly related to those across toad bladder, although there does exist a profound difference in structure. Recently, Stephan and his coworkers (1959a,b; 1960; 1961) have shown that the inhibition of thyroid secretion results in a decrease in the resorption processes for both ions and water which normally occur in the kidney. Restoration of the thyroid levels within the hypothyroid rat results in more normal levels of excretion of water and ions. This may be compared to the present <u>in vitro</u> studies where thyroxine, applied to tissues washed free of endogenous hormone, causes increased transfer of ions and water across the bladder. If the analogy between the more structurally simple bladder and the kidney tubule is correct, then we may make direct comparsons between these two sets of results. The Amphibian bladder serves

as a storage for urine and ions are resorbed into the body from the mucosal to the serosal surfaces. It is known that the mammalian kidney tubule resorbs ions and water from the mucosal to the serosal surface (Giebisch, 1962), for the mammalian bladder serves only as a storage organ. Similar processes, therefore, occur in the same direction, i.e. into the plasma, across both membranes. Since thyroxine results in increased transfer of electrolytes and water across the Amphibian bladder it is possible to infer that similar processes may be accelerated by thyroxine across the mammalian kidney tubule. The in vivo work performed in the present study shows that such resorptive processes are accelerated even after injection of thyroxine into the whole animal, although localisation of the effect is impossible under the conditions of the experiement. Although fish are faced with a different osmotic problem than Amphibia, the results obtained by Hickman (1959) are of importance. Upon transference of fish to environments of varying salinity, he noticed an increase in thyroid activity as well as a concurrent increase in ionic regulation. Occurring concurrently with these two processes was an increase in metabolism. The energy demands are greater in more saline environments and the quantitative differences in ion transport appear to be the origin of the greater demand for thyroid hormone. Although it is not known where the thyroid hormone exerts its influence in osmoregulation, it is likely that it stimulates oxidative metabolism of the cells doing osmotic work.

The effects of other hormones, such as the antidiuretic hormone

and corticosteroids, on ion balance in Amphibia, are well known and the effect of thyroxine injection is quantitatively much smaller than that produced by either of these other substances. It has been shown, for instance, that in the hypophysectomised frog the water balance response to vasopressin is restored to near-normal by adrenocorticotrophic hormone (ACTH). Restoration of the water balance response to absolute normal was achieved by injection of both ACTH and thyroxine (Levinsky and Sawyer, 1952). This work demonstrates that although hormones may have their own individual effects on various parameters of activity, the complete spectrum of hormones normally present in the whole animal is necessary for the fulfillment of normal body processes.

The present study has, therefore, shown that thyroxine and triiodothyronine contribute to the control of both cellular and tissue activity within the living vertebrate. The in vitro studies, although limited in the parameters of activity measured, do offer, however, some indication of how the thyroid hormones exert their effects within animal tissues. The studies also offer an approach towards a further understanding of thyroid hormone action on the structure and function of the cell, although by no means providing a complete understanding of their action. One remarkable factor as regards the thyroid hormones is the constancy of molecular structure throughout the vertebrate series. This, however, appears to be no barrier to their involvement in a great variety of adaptive responses seen in different groups (Barrington, 1962). Thus the hormones appear to control some overall cellular

process, such as permeability, rather than a specific process for specific 'receptors', such as enzymes, would vary in different animals. It is easily seen, furthermore, that alterations in permeability could bring about changes in exchange of substances across the cell wall, thus providing more substrate for the particular enzymes present in the different groups. Thus the profound changes which are seen after thyroid treatment could be brought about without the necessity of postulating an effect on one or more 'pacemakers', the presence of which would be necessary in each group. Together with other work on hormone action these studies indicate that it is impossible, as yet, to understand completely how the interplay between different hormones brings about their manifold effects within the body of the living vertebrate.

During the course of this work the following publications have been presented, Green and Matty (1962; 1963a,b,c) and Matty and Green (1962a,b; 1963a,b,c), which outline the temporal development of the theories embodied in this thesis.

SUMMARY AND CONCLUSIONS

Several techniques have been outlined for the measurement of various parameters of activity of Amphibian skin and bladder *in vitro*. The effects of the thyroid hormones, L-thyroxine and 3;5;3' -triiodo-L-thyronine, as well as several analogues on these cellular processes have been monitored. From these measurements the following results have been obtained:-

a) Thyroxine and triiodothyronine in concentrations of 10^{-8}M to 10^{-6}M were shown to increase water transfer down an osmotic gradient in the isolated toad bladder. The increase after hormonal treatment was related linearly to the log dose, but addition of thyroxine resulted in a greater maximal value and a greater total loss than that of triiodothyronine. Triiodothyronine, however, had a much shorter 'latent' period than thyroxine.

b) The hormones also enhance water movement across the bladder under iso-osmotic conditions and a correlation has been shown between this and the effect of the hormones on active cation transport.

c) Both hormones, when applied to the serosal surface of either the skin or bladder of the toad, cause an increase in active sodium transport within one hour of application. This has been measured both electrically and isotopically. Permeability to anions has been shown to be passive and it is concluded that this increased anion, particularly chloride ion, permeability is the cause of the 'dynamic' stability of the potential under treated conditions.

d) The hormones result in an increase in oxygen uptake of the

tissues when applied *in vitro* and this implies a metabolic role of the compounds in adult Amphibia. These results also imply an effect, either direct or indirect, on enzyme systems within the cell. It has been shown that this rise in respiration greatly exceeds that required by the increased, energy-dependent process of active sodium transport. Thus, thyroxine not only accelerates the phenomena involved in the transport process, but also stimulates those enzymatic processes concerned directly or indirectly with general respiratory metabolism of the cell.

e) Substitution of sodium ions by choline in the medium results in a fall of oxygen uptake and also a slight lowering of water loss When hormone was added to the isolated tissues oxygen uptake increased to a lesser extent than when added in sodium medium, whilst the water-loss increase was also reduced. Specific enzyme inhibitors reduced the hormone-induced increases in water loss from the bladder, thereby indicating that both glycolytic and oxidative energy is necessary for the alteration in permeability.

f) Tissue ion content under hormonal treatment indicates that the compounds cause profound alterations in ion movement, which are reflections of their effects on ion movement across cellular membranes. Changes in tissue sodium and potassium content slightly precede metabolic alterations and it is evident that certain primar effects precede more profound alterations in permeability.

g) A mixture of thyroxine and triiodothyronine brought about changes in all the parameters measured which showed a direct relationship to those seen when both substances were added independently. The 'thyroxine' part of the curve was, however, moved

nearer to the point of addition of the hormonal solution once mo indicating a permeability effect, this time of triiodothyronine upon thyroxine entry into cells.

h) From the results it appears that triiodothyronine penetrate cells at a faster rate than does thyroxine and in addition is mo rapidly metabolised.

i) None of the analogues used had any effect on any parameters, save that of TETRAC and TRIAC on water loss across the bladder down an osmotic gradient. This result is indicative of biologic activity, but the lack of effect on any other parameter tends to argue against this concept. The results show that the peripher-ally active substances in Amphibia are in fact the parent hormone

j) In an attempt to correlate the *in vitro* findings with *in viv* hormone activity, experiments were performed on whole animals. Injections of thyroxine into *Bufo bufo* cause a net cutaneous up-take of salt, compared to the net loss observed before treatment. Urinary volume and the total excretion of sodium and chloride were reduced after thyroxine treatment, thus the urine ionic concen-tration was decreased. Weight loss during the first seven days of thyroxine administration again argues for a metabolic effect of the hormone. A relationship is suggested between the *in vitro* work, the *in vivo* study and the study of moulting where it is known that thyroxine has a role. It may be that thyroxine is responsib for the changes in electrolyte balance seen at the time of moulting

The above results enable an hypothesis of thyroid hormone action to be proposed. It is postulated that thyroxine and trii-odothyronine initially affect the primary cell membrane and re-

sult in alterations of the inorganic and organic substrate supply of the cell. This phase is followed by a secondary effect, which is an alteration in enzymic activity (itself partly a result of the primary effect), and this further brings about the more profound effects on permeability, such as alterations in 'pore' size and cellular geometry. In addition, the hormones must also exert some direct effect on enzymes, for studies by several workers have shown that vasopressin causes alterations in pore size without any concurrent effect on metabolism. The thyroid hormones, however, stimulate basal respiration even in the absence of sodium, thus a direct effect on enzymes is a necessary postulate. Direct evidence for this is lacking in the present experiments, although the metabolic response argues for an enzymic effect.

The present study illustrates a new mode of approach for the analysis of thyroid hormone action and presents a detailed study of the action of the hormones on ion movements and the metabolism of the tissues. The work has also shown that the thyroid hormones control the permeability of cells as well as exerting intracellular effects either directly or indirectly - as a result of the membrane effects. As such, the study brings the thyroid hormones into a fresh light for, although they have been shown to increase amino-acid incorporation, regulation of ion movement at the cellular or membrane level has not previously been investigated. The *in vivo* work has confirmed the *in vitro* findings and shown them to be a physiological reality. Although by no means offering a complete understanding of the hormone activity at the cellular level,

the work does offer a stepping stone to more detailed and fundamental investigations on the cell. Microelectrode studies, used in conjunction with isotopic measurements, would yield valuable information as to the specific locus of action within the cells and provide an analysis of each step of the action.

REFERENCES

Adams, A.E. and Gray, B. (1936). A comparative study of the thyroid glands of hypophysectomised newts after treatment with anterior pituitary, thyroid and iodine. Anat. Rec., 65, 69.

Ahlgren, G. (1925). Zur Kenntnis der tierischen Geweboxydatio sowie ihrer Beinflussung durch Insulin, Adrenalin, Thyroxin und Hypophyseprarate. Skand. Arch. Physiol., 47, suppl. 225.

Ahmed, K., Judah, J.D. and Wallgren, H. (1963). Phosphoprotei and ion transport of cerebral cortex slices. Biochi biophys. Acta, 69, 428.

Albright, E.C., Larson, F.C., Tomita, K. and Lardy, H.A. (1956) Enzymatic conversion of thyroxine and triiodothyroni to the corresponding acetic analogues. Endocrinolog 59, 252.

Alexander, W.D. and Bisset, S.K. (1958). Immediate effects of triiodothyroacetic acid on oxygen consumption of mye loid leukemic leucocytes in vitro. Lancet, ii, 1265

Althausen, T.L. (1949). Hormonal and vitamin factors in intest nal absorption. Gastroenterology, 12, 467.

Anderson, B. (1959). quoted by Ussing, H.H. Active and passive transport across epithelial membranes. In,'The Metho of Isotopic Tracers Applied to the Study of Active Io Transport'. J. Coursaget, ed. p.139. Pergamon Press, London.

Anderson, W.A. and Brown, E. (1963). The influence of arginine vasopressin upon the production of adenosine 3',5'- monophosphate by adenyl cyclase from the kidney. Biochim. biophys. Acta, 67, 674.

Angerer, C.A. (1950). Body weight, survival times, coloration and water content of skeletal muscles of adrenalecto- mised frogs. Ohio J.Sci., 50, 103.

Aschkenasy, A. (1963). Effects de la thyroxine, de la cortisone et des androgenes sur la reticulocytose sanguine et sur l'incorporation du 59Fe dans les erythrocytes et dans certains organes chez des Rats carences en pro- teines. C.R. Soc. Biol., 157, 91.

Asher(quoted by Loeb, L.). (1923). Edema. Medicine, 2, 171.

Asper, S.P., Selenkow, H.A. and Plamondon, C.A. (1953). A comparison of the metabolic activities of 3;5;3'-triiodo-L-thyronine and L-thyroxine in myxedema. Johns Hopk. Hosp Bull., 93, 164.

Aulsebrook, K.A. (1961). Effect of vasopressin on sodium transfer by rat colon in vitro. Endocrinology, 68, 1063.

Bakke, J. L., Heideman, M.L., Lawrence, N.L. and Wiberg, C. (1957) Bioassay of thyrotropic hormone by weight response of bovine thyroid slices. Endocrinology, 61, 352.

Baldwin, E. (1959). Dynamic aspects of biochemistry, 3rd. ed. Cambridge University Press.

Baraduc, M.M. (1957). Influence de la thyroxine sur les exchanges de l'ion chlore de la Truite arc-en-ciel, (Salmo gairdneri). C.R. Soc. Biol., 151, 303.

Barch, S.H. (1953). Oxygen consumption of normal and thyroxine stimulated Rana pipiens skin. Physiol. Zool., 26, 223.

Barker, S.B. (1955). Thyroid. Ann. Rev. Physiol., 17, 417.

Barker, S.B. (1956). Metabolic actions of thyroid derivatives and analogues. Endocrinology, 59, 548.

Barker, S.B. (1962). Peripheral actions of thyroid hormones. Fed. Proc., 21, 635.

Barker, S.B. and Lewis, W.J. (1956). Metabolic actions of acetic acid analogues of thyroxine and triiodothyronine. Proc. Soc. exp. Biol. Med. N.Y., 91, 650.

Barrington, E.J.W. (1962). Hormones and vertebrate evolution. Experientia. 18, 1.

Barrnett, R.J. and Ball, E.G. (1960). Metabolic and ultrastructural changes induced in adipose tissue by insulin. J. Biophys. biochem. Cytol., 8, 83.

Beigelman, P.M. and Hollander, P.B. (1962). Effect of insulin upon resting electrical potential of adipose tissue. Proc. Soc. exp. Biol. Med. N.Y., 110, 590.

Beisel, W.R., Zerzan, C.J., Rubini M.E. and Blythe, W.B. (1958). Phosphaturesis; A direct renal effect of triiodothyronine. Am.J. Physiol., 195, 357.

Bentley, P.J. (1958). The effects of neurohypophyseal extracts on water transfer across the wall of the isolated urinary bladder of the toad, Bufo marinus. J. Endocrin., 17, 201.

Bentley, P.J. (1960). The effects of vasopressin on the short-circuit current across the wall of the isolated bladder of the toad, Bufo marinus. J. Endocrin., 21, 161.

Bentley, P.J. (1962). Studies on the permeability of the large intestine and urinary bladder of the tortoise (Testudo Graeca) with special reference to the effects of neurohypophyseal and adrenocortical hormone. Gen. Comp. Endocrin., 2, 323.

Bentley, P.J. and Follett, B.K. (1962). The action of neurohypophyseal and adrenocortical hormones on sodium balance in the cyclostome, Lampetra fluviatilis. Gen. Comp. Endocrin., 2, 329.

Bishop, W.R., Mumbach, M.W. and Scheer, B.T. (1961). Interrenal control of active sodium transport across frog skin. Am.J. Physiol., 200, 451.

Bisset, S.K. and Murray, I.P.C. (1962). The stimulation of oxygen uptake by myxoedematous leucocytes in vitro by triiodothyroacetic acid. Biochem. J., 84, 17P.

Bitman, J., Cecil, H.C., Hawk, H.H. and Sykes, J.F. (1959). Effect of oestrogen and progesterone on water and electrolyte content of rabbit uteri. Am. J. Physiol., 197. 93.

Boekelman, W.A. (1958). Sodium content of erythrocytes in hyperthyroidism. Nature, 181, 1136.

Bonting, S.L. and Caravaggio, L.L. (1963). Studies on sodium-potassium-activated adenosinetriphosphatase. V. Correlation of enzyme activity with cation flux in six tissues. Arch. Biochem. Biophys., 101, 37.

Brande, P.F. and Knobil, E. (1962). Further evidence for amino acid transport as a site of action of growth hormone. Proc. Soc. exp. Biol. Med. N.Y., 110, 15.

Bransome, E.D. and Reddy, W.J. (1963). Hormonal effects in vitro on amino acid incorporation into rat adrenal protein; adrenocorticotrophin and growth hormone. Arch. Biochem. Biophys., 101, 21.

Bricker, N.S., Biber, T. and Ussing, H.H. (1963). Exposure of the isolated frog skin to high potassium concentrations at the internal surface. I. Bioelectric phenomena and sodium transport. J. Clin. Invest., 42, 88.

Brown, A.C. (1962). Current and potential of frog skin in vivo and in vitro. J. Cell. Comp. Physiol., 60, 263.

Brown, W.O. and Badman, H.G. (1961). Liquid scintillation counting of ^{14}C labelled animal tissues at high efficiency. Biochem. J., 78, 571.

Bruhn, J.M. (1941). The relation of heat production to water metabolism during the administration of synthetic thyroxine. Am. J. Physiol., 135, 1572.

Bruice, T.C., Winzler, R.J. and Kharasch, N. (1954). The thyroxine-like activity of some new thyroxine analogues in Amphibia. J. Biol. Chem., 210, 1.

Brull, L. (1940). The site of action of thyroxine on water metabolism. Quart. J. exp. Physiol., 30, 195.

Byrom, F.B. (1933-34). The nature of myxoedema. Clin. Sci., 1, 273.

Caldwell, P.C. (1959). Quelques relations entre le metabolism et les transport actifs d'ions dans l'axone geant du Calmar. In 'The Method of Isotopic Tracers Applied to the Study of Active Ion Transport'. J. Coursaget, ed. p.88 Pergamon Press, London.

Caldwell, P.C. and Keynes, R.D. (1957). The utilization of phosphate bond energy for sodium extrusion from giant axons J. Physiol., 137, 12P.

Canessa-Fischer, M., Edelman, C.M. and Davis, R.P. (1962). The coupling of ion transport and energy metabolism in the toad bladder. Fed. Proc., 21, 148.

Capraro, V. and Marro, F. (1963). The net passage of water through the isolated skin of Rana asculenta in the absence of apparent osmotic gradient. Arch. Ital. Biol., 101, 161.

Cereijo-Santalo, R., Di Nella, R., Park, C.R., Park, J.H. and Pitt-Rivers, R. (1958). Effect of thyroxine analogues on serobic glycolysis and oxygen consumption of ascites tumor cells. Fed. Proc., 17, 784.

Chance, B. and Williams, G.R. (1955). A simple and rapid assay of oxidative phosphorylation. Nature, 176, 250.

Chavin, W. and Rassmoore, H.W. (1956). Pituitary-thyroid regulation of respiration in Carrasius auratus L. Anat. Rec., 125, 599.

Chester Jones, I. (1957). The Adrenal Cortex. Cambridge University Press.

Clark, N.B. and Kaltenbach, J.C. (1961). Direct action of thyroxine on skin of adult newt. Gen. Comp. Endocrin., 1, 513.

Cole, K.S. and Curtis, H.J. (1938a). Electrical impedence of Nitella during activity. J. Gen. Physiol., 22, 37.

Cole, K.S. and Curtis, H.J. (1938b). Electrical impedence of the squid giant axon during activity. J. Gen. Physio 22, 649.

Comsa, J. (1957). The effect of thyroxine on glucose utilisati by the isolated rat diaphragm. Experientia, 13, 499

Connelly, C.M. (1957). Methods for measuring tissue oxygen tension. Theory and evaluation; The oxygen electrode. Fed. Proc., 16, 681.

Conway, E.J. (1953). A redox pump for the biological performanc of osmotic work, and its relation to the kinetics of free ion diffusion across membranes. Intern. Rev. Cytol., 2, 419.

Corcoran, A.C. and Page, I.H. (1947). Specific renal functions in hyperthyroidism and myxoedema: effects of treatment. J. Clin. Endocrin., 7, 801.

Crabbe, J. (1961a). Stimulation of active sodium transport across the isolated toad bladder after injection of aldosterone to the animal. Endocrinology, 69, 673.

Crabbe, J. (1961b). Stimulation of active sodium transport by the isolated toad bladder with aldosterone in vitro. J. Clin. Invest., 40, 2103.

Crane, E.E. and Davies, R.E. (1951). Chemical and electric energy relations for the stomach. Biochem. J., 49, 169

Crane, R.K. (1962). Hypothesis for mechanism of intestinal active transport of sugars. Fed. Proc., 21, 891.

Creese, R., D'Silva, J.L. and Northover, J. (1958). Effect of insulin on sodium in muscle. Nature, 181, 1278.

Csaky, T.Z. and Fernald, G.W. (1961). Localization of the 'sugar pump' in the intestinal epithelium. Nature, 191, 709.

Curran, P.F. (1960). Na, Cl and water transport across the rat ileum in vitro. J. Gen. Physiol., 43, 1137.

Curran, P.F., Herrera, F.C. and Flanigan, W.J. (1963). The effect of Ca and antidiuretic hormone on Na transpor across frog skin. J. Gen. Physiol., 46, 1011.

Curran, P.F. and MacIntosh, J.R. (1962). A model system for b logical water transport. Nature, 193, 347.

Curran, P.F. and Schwartz, G.F. (1960). NaCl and water transp by rat colon. J. Gen. Physiol., 43, 555.

Curran, P.F. and Solomon, A.K. (1957). Ion and water fluxes i the ileum of the rat. J. Gen. Physiol., 41, 143.

Dallam, R.D. and Howard, R.B. (1960). Thyroxine-enhanced oxida tive phosphorylation of rat liver mitochondria. Biochem. biophys. Acta, 37, 188.

Davenport, H.W. and Chavre, V.J. (1952). Evidence that glycol- ysis contributes to gastric acid secretion. Am. J. Physiol. 171, 1.

Davies, J.E., Da Costa, E. and Hastings, B. (1934). The effect of thyroxine on the tissue metabolism of excised frog heart. Am. J. Physiol, 110, 187.

Dickens, F. and Salmony, D. (1956). Effects of thyroid hormone in vitro on tissue respiration, oxidative phosphory- lation and the swelling of mitochondria. Biochem. J. 64, 645.

Dodd, J.M. and Matty, A.J. (1963). Comparative aspects of thy- roid function. The thyroid. Vol. 1. J. & A. Butter- worths Ltd., London. In Press.

Donn, A., Rosenblatt, M.G. and Christy, N.P. (1961). Effect of aldosterone on the living cornea in vitro. Nature, 192, 73.

Donoso, A.O. (1960). Action des hormones thyroidiennes sur le metabolisme des tissues isoles de crapaud. C.R. Soc. Biol., 154, 832.

Donoso, A.O. and Trivelloni, J.C. (1958). Hormones tiroideas y metabolismo del sapo. Rev. Soc. Argent. Biol., 34, 46.

Drexler, E. and von Issekutz, B. (1935). Die Wirkung des Thyro- xine auf den Stoffwechsel kaltblutiger Wirbeltiere. Arch. exp. Path. Pharmak., 177, 435.

Durbin, R.P., Curran, P.F. and Solomon, A.K. (1958). Ion and water transport in stomach and intestine. Adv. in Biol. Med. Phys., 6, 1.

Eichhorn, J., Feinstein, M., Halkerston, I.D.K. and Hechter,

O. (1961). Effects of corticoids, insulin and epinephrine on aminoisobutyrate accumulation in muscle of adrenalectomised rats. Proc. Soc. Exp. Biol. Med. N.Y. 106, 781.

Embden, G. and Adler, E. (1922). Uber die physiologische Bedeutung des Weschels des Permeabilitats zustardes von Muskelfasergrenzschichten. Z. f. physiol. Chem., 118, 1.

Engbaek, L. and Hoshiko, T. (1957). Electrical potential gradient through the frog skin. Acta. Physiol. Scand., 39, 348.

Ermans, A.M. (1960). Action de l'hormone thyroidienne sur la vitesse de remplacement du phosphate acidosoluble des globules rouges in vivo. 1° Internat. Congr. Endocrin., Copenhagen. p. 1223.

Ernster, L., Siekevitz, P. and Palade, G.E. (1962). Enzyme-structure relationships in the endoplasmic reticulum of rat liver. J. Cell Biol., 15, 541.

Essig, A., Frazier, H.S. and Leaf, A. (1963). Evidence for 'electrogenic' active sodium transport across an epithelial membrane. Nature, 197, 706.

Etling, N. and Barker, S.B. (1959). Metabolism of thyroxine during prolonged kidney cortex incubation. Endocrinology, 64, 753.

Ewer, R. (1951). Water uptake and moulting in Bufo regularis Reuss. J. exp. Biol., 28, 369.

Ewer, R. (1952a). The effect of Pituitrin on fluid distribution in Bufo regularis Reuss. J. exp. Biol., 29, 173.

Ewer, R. (1952b). The effects of posterior pituitary extracts on water balance in Bufo carens and Xenopus laevis, together with some general considerations of Anuran water economy. J. exp. Biol., 29, 429.

Fairhurst, A.S., Roberts, J.C. and Smith, R.E. (1959). Effects of physiological levels of thyroxine in vivo on respiration and phosphorylation in rat liver fractions. Am. J. Physiol., 197, 370.

Fisher, R.B. (1955). The absorption of water and of some small solute molecules from the isolated small intestine of the rat. J. Physiol., 130, 655.

Fontaine, M. (1956). The hormonal control of water and salt electrolyte metabolism in fish. Mem. Soc. Endocrin., 5, 69.

Forte, J.G., Adams, P.H. and Davies, R.G. (1963). Source of the gastric mucosal potential difference. Nature, 197, 874

Fowler, M.A. quoted by Chester Jones, I. 'The Adrenal Cortex'. 1957. Cambridge University Press.

Francis, G.E., Mulligan, W. and Wormall, A. (1959). Isotopic Tracers. 2nd. Edn. The Athlone Press, University of London.

Frazier, H.S. (1962). The electrical potential profile of the isolated toad bladder. J. Gen. Physiol., 45. 515.

Frazier, H.S. and Hammer, E.I. (1963). Efflux of sodium from isolated toad bladder. Am. J. Physiol., 205, 718.

Frazier, H.S. and Leaf, A. (1963). The electrical characteristics of active sodium transport in the toad bladder. J. Gen. Physiol., 46, 491.

Fregley, M.J., Brimhall, R.L. and Golindo, O.J. (1962). Effect of the antithyroid drug, propylthiouracil, on the sodium balance of rats. Endocrinology, 71, 693.

French, I.T. and Manery, J.F. (1962). The effect of aldosterone on tissue electrolytes. Fed. Proc., 21, 186.

Frieden, E. and Westmark, G.W. (1961). On the anomalous activity of thyroxine analogues in tadpole. Science, 133, 463.

Frienkel, N., Ingbar, S.H. and Dowling, J.T. (1953). The interaction of thyroxine with plasma proteins; localization of thyroxine-binding protein in Cohn fractions of plasma. J. Clin. Invest., 34, 1698.

Fromm, P.O. and Reineke, E.P. (1956). Some aspects of thyroid physiology in rainbow trout. J. Cell. Comp. Physiol., 48, 393.

Fuhrman, F.A. and Ussing, H.H. (1951). A characteristic response of the isolated skin potential to neurohypophyseal principles and its relation to the transport of sodium and water. J. Cell. Comp. Physiol., 38, 109.

Galton, V.A. and Ingbar, S.H. (1962). Observations on the relation between the action and degredation of thyroid hormones as indicated by studies in the tadpole and the frog. Endocrinology, 70, 622.

Galton, V.A. and Ingbar, S.H. (1962b). Observations on the effec and the metabolism of thyroid hormones in Necturus maculosus. Endocrinology, 71, 369.

Galton, V.A. and Pitt-Rivers, R. (1959). The identification of the acetic acid analogues of thyroxine and triiodothyronine in mammalian tissues. Biochem. J., 72, 319.

Gatzy, J. and Clarkson, T.W. (1962). Relative ionic permeabilities of the mucosal and serosal surfaces of the isolated toad bladder. Fed. Proc., 21, 149.

Gaunt, R. (1944). The effect of thyroxine on water diuresis and water intoxication in the rat. Fed. Proc., 3, 12.

Gayda, T. (1922). Contributo allo studio della fisiologica della tiroide della Rana. Arch. Fisiol., 20, 209.

Gellhorn, E. (1929). Das Permeabilitats problem, seine physiologische und allgemein-pathologische Bedeutung. Berlin.

Gellhorn, E. and Northrup, D. (1933). Quantative investigations on the influence of hormones on absorption. II. Internal secretions and permeability. Am. J. Physiol., 103, 382.

Gemmill, C.L. (1940). The effect of insulin on the glycogen content of isolated muscles. Johns Hopk. Hosp. Bull., 66, 232.

Gemmill, C.L. (1941). The effects of glucose and of insulin on the metabolism of the isolated rat diaphram. Johns. Hopk. Hosp. Bull., 68, 329.

Genest, A.A. and Adams, A.E. (1957). The daily thyroxine requirements of the adult male Triturus viridescens. Anat. Rec., 127, 418.

Gennes, L. de. and Bricaire, H. (1951). Le role des glandes endocrines dans la regulation du metabolism de l'eau. 3 Congres Medical Internat. D'Evian, p. 115.

Giebisch, G. (1962). Kidney, water and electrolyte metabolism. Ann. Rev. Physiol., 24, 357.

Gimeno, A.L., Gimeno M. and Webb, J.L. (1963). Sex hormones studies. The effect on the cellular membrane potentials and contractility of isolated rat atrium. Calif. Med., 98, 67.

Glynn, I.M. (1956). Sodium and potassium movements in human red cells. J. Physiol., 134, 278.

Goh, K. and Dallam, R.D. (1957). Oxygen consumption of the auricles, right and left ventricles of normal, hypothyroid and hyperthyroid rat heart. Am. J. Physiol., 188, 514.

Goldacre, R.J. (1952). The folding and unfolding of protein molecules as a basis of osmotic work. Intern. Rev. Cytol., 1, 135.

Goodfriend, T. and Kirkpatrick, J. (1963). Effects of neurohypophyseal hormones on oxidative metabolism of the toad bladder *in vitro*. Endocrinology, 72, 742.

Gorbman, A. and Ueda, K. (1963). Electrical properties of thyroid follicles in normal and thyrotropin-injected frogs and in premetamorphic and metamorphic tadpoles. Gen. Comp. Endocrin., 3, 308.

Green, D.E. (1941). Enzymes and trace substances. Adv. Enzymol., 1, 177.

Green, D.E. (1957). Orginization in relation to enzymic function. In 'Mitochrondia and Other Cytoplasmic Inclusions'. S.E.B. Symposium No. 10, p. 30. Cambridge University Press.

Green, K. and Matty, A.J. (1962). The effect of thyroxine on the permeability of the isolated toad bladder. Nature, 194, 1190.

Green, K. and Matty, A.J. (1963a). Action of thyroxine on active sodium transport in isolated membranes of Bufo bufo. Gen. Comp. Endocrin. 3, 244.

Green, K. and Matty, A.J. (1963b). The influence of thyroxine on sodium, chloride and water balance of the toad, Bufo bufo. Gen. Comp. Endocrin., 3, 446.

Green, K. and Matty, A.J. (1963c). The effect of thyroidal hormones on water permeability of the isolated bladder of the toad, Bufo bufo. J. Endocrin., 28, In press.

Green, K., Seshadri, B. and Matty, A.J. (1962). Independence of transfer of solute and solvent across the rat ileum. Nature, 196, 1322.

Greif, R.L. and Moroney, J. (1959). A comparison of the effects of *in vivo* and *in vitro* thyroid hormones upon the oxygen consumption of rat kidney slices. Endocrinology, 64, 937.

Gross, J. and Pitt-Rivers, R. (1951). Unidentified iodine compounds in human plasma in addition to thyroxine and iodine. Lancet, 11, 766.

Gross, J. and Pitt-Rivers, R. (1953). 3;5;3'-triiodothyronine. II Physiological activity. Biochem. J., 53, 652.

Haarman, W. (1936). Uber den Einfluss von Thyroxin auf den Sauerstoffverbrauch uberlebender Gewebe. Arch. exp. Path. Pharmak., 108, 167.

Hakkila, J., Siltanen, P.K., Miettinen, O.S. and Karki, N.T. (1962 Immediate effects of triiodothyroacetic acid on isolated perfused rabbit and guinea pig hearts. Ann. Med. Exp. Fenn., 40, 76.

Hamolsky, M.W., Michel, R., Carnicero, M. and Roche, J. (1963). The effects of thyroid hormones on oxygen consumption of isolated horse leucocytes. Biochem. biophys. Acta, 69, 420.

Harington, C.R. (1926). Chemistry of thyroxine. I, Isolation of thyroxine from the thyroid gland. Biochem. J., 20, 293

Harington, C.R. (1927). Chemistry of thyroxine. II. Constitution and synthesis of thyroxine. Biochem. J., 21, 169.

Haynes, R.C., Sutherland, E.W. and Rall, T.W. (1960). The role of cyclic adenylic acid in hormone action. Rec. Progr. Hormone Res., 16, 121.

Hays, R.M. and Leaf, A. (1961). The problem of clinical vasopressin resistance; in vitro studies. Ann. Int. Med., 54, 700.

Hays, R.M. and Leaf, A. (1962a). Studies on the movement of water through the isolated toad bladder and its modification by vasopressin. J. Gen. Physiol., 45, 905.

Hays, R.M. and Leaf, A. (1962b). The state of water in the isolated toad bladder in the presence and absence of vasopressin. J. Gen. Physiol., 45, 933.

Hechter, O. (1955). Concerning possible mechanisms of hormone action. Vitamins and Hormones, 13, 293.

Hechter, O. and Lester, G. (1960). Cell permeability and hormone action. Rec. Prog. Hormone Res., 16, 139.

Heinz, E. (1963). Physiologie, Biochemie and Energetik des aktiven Transports. Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak., 245, 10.

Heller, H. (1930). Uber die einwirkung von Hypophysen hinterlappen extracten auf den Wasserhaushalt des Frosches. Arch. exp. Path. Pharmak. 157, 298.

Heller, H. (1945). Effect of neurohypophyseal extracts on water balance. Biol. Rev., 20, 147.

Heller, H. (1950). The comparative physiology of the neurohypophysis. Experientia, 6, 368.

Heller, H. (1956). The hormonal control of water and salt electrolyte metabolism with special reference to higher vertebrates. Mem. Soc. Endocrin., 5, 25.

Heller, H. (1957). The metabolism and fate of the neurohypophyseal principles. In 'The Neurohypophysis', H. Heller, ed. p. 77. J. & A. Butterworths Ltd., London.

Henschel, H. and Steuber, M. (1931). Uber die Bedeutung der Schildrusse fur den Stoffwechsel der Amphibian. Arch. exp. Path. Pharmak. 160, 401.

Herrera, F.C., Whittembury, G. and Planchart, A. (1963). Effect of insulin on short circuit-current across isolated frog skin in presence of calcium and magnesium. Biochem biophys. Acta, 66, 170.

Hevesy, G.V., Hofer, E. and Krogh, A. (1935). The permeability of the skin of frogs to water as determined by D_2O and H_2O. Scand. Arch. Physiol., 72, 199.

Hickman, C.P. (1959). The osmoregulatory role of the thyroid gland in the starry flounder, Platichthys stellatus. Can. J. Zool., 37, 997.

Hill, D.K. (1948). Oxygen tension and respiration;frog muscle. J. Physiol. 107, 479.

Hoch, F.L. (1962). Biochemical actions of thyroid hormones. Physiol. Revs., 42, 605.

Hokin, L.E. and Hokin, M.R. (1961). Studies on the enzyme mechanism of the sodium pump. In 'Membrane Transport and Metabolism', A. Kleinzeller and A. Kotyk, eds. p. 204. Academic Press. N.Y.

Holmes, W.N. (1959). Studies on the hormonal control of sodium metabolism in the rainbow trout (Salmo gairdneri). Acta Endocrin., Copenhagen, 31, 587.

Holmes, W.N. and Butler, D.G. (1963). The effect of adrenocortical steroids on the tissue electrolyte composition of the fresh-water rainbow trout (Salmo gairdnerie). J. Endocrin., 25, 457.

Holmes, W.N., Phillips, J.G. and Chester Jones, I. (1963). Adrenocortical factors associated with adaption of vertebrates to marine environments. Rec. Progr. Hormone. Res., 19, 619.

Hoover, C.R. and Turner, C.W. (1954). In vitro metabolism of

the rat mammary gland and observations on *in vitro* actions of thyroid hormones. Univ. of Missouri Coll. Agric. Res. Bull. No. 563 p. 50.

Hoshiko, T. and Ussing, H.H. (1960). The kinetics of ^{24}Na flux across amphibian skin and bladder. Acta Physiol. Scand., 49, 74.

House, C.R. and Green, K. (1963). Sodium and water transport across isolated intestine of a marine teleost. Nature, 199, 1293.

Hsu, K. and Oliner, L. (1962). Effect of sodium-L-Thyroxine on sodium and potassium in yeast. Fed. Proc., 21, 146.

Huf, E. (1935). Versuche Uber den Zusamenhang zwischen Stoffwechsel, Potentialbildung, und Function der Froschhaut. Pflug. Arch. ges. Physiol., 235, 655.

Huf, E. (1936). Ueber aktiven Wasser-und Salz-transport durch die Froschhaut. Pflug. Arch. ges. Physiol., 237, 143.

Huxley, A.F. (1959). Ion movements during nerve activity. Ann. N.Y. Acad. Sci., 81, 221.

Jacob, A. (1962). Action immediate de la thyroxine sur les oxydations du tissue hepatique *in vitro*. I. Influence de quelques facteurs physiologiques. Arch. Sci. Physiol., 16, 413.

Jahn, H., Reville, P. and Stephan, F. (1962). Diminution de la reabsorption tubulaire du sodium chez le Rat hypothyroidienne au cours de la polyurie osmotique au mannitol. C.R. Socl Biol., 156, 1927.

Jard, S., Maetz, J. and Morel, F. (1960). Action de quelques analogues de l'ocytocine sur difference recepteurs intervenant dans l'osmoregulation de *Rana esculents*. C.R. Acad. Sci., 251, 788.

Jarnefelt, J. (1962a). Properties and possible mechanism of the Na and K -stimulated microsomal adenosinetriphosphatase. Biochim. biophys. Acts, 59, 643.

Jarnefelt, J. (1962b). Some aspects of the physiological significance of the adenosinetriphosphatase of brain microsomes. Biochim. biophys. Acta, 59, 655.

Johnson, P.C. and Redding, T. (1961). A possible site of action of thyroid hormone. J. Lab. Clin. Med., 58, 832.

Jørgensen, C.B. (1947). Influence of adenohypophysectomy on the transfer of salt across the frog skin. Nature, 160, 872.

Jørgensen, C.B. (1949). Permeability of the Amphibian skin. II. Effect of moulting of the skin of Anurans and the permeability to water and electrolytes. Acta Physiol. Scand., 18, 171.

Jørgensen, C.B. (1950). The Amphibian water economy, with special regard to the effect of neurohypophyseal extracts. Acta Physiol. Scand., 22, suppl. 78.

Jørgensen, C.B. (1954). On excretion of chloride in sodium chloride loaded frogs and toads. Acta Physiol. Scand., 30, 171.

Jørgensen, C.B. and Larsen, L.O. (1960). Hormonal control of moulting in Amphibia. Nature, 185, 244.

Jørgensen, C.B. and Larsen, L.O. (1961). Moulting and its hormonal control in toads. Gen. Comp. Endocrin., 1, 145.

Jørgensen, C.B., Levi, H. and Ussing, H.H. (1949). On the influence of the neurohypophyseal hormones on the sodium metabolism in the axolotl, (Amblystoma mexicanum). Acta Physiol. Scand., 12, 356.

Judah, J.D. and Ahmed, K. (1962). Sodium transport and phosphoproteins. Nature, 194, 382.

Judah, J.D., Ahmed, K. and McLean, A.E.M. (1962). Phosphoproteins and sodium transport. Nature, 196, 484.

Kallman, H. and Furst, M. (1958). The basic processes occuring in the liquid scintillator. In 'Liquid Scintillation Counting', C.G. Bell and F.N. Haynes, eds. p. 3. Pergamon Press, London.

Kalman, S.M. and Ussing, H.H. (1954). Active sodium uptake by the toad, and its response to the antidiuretic hormone. J. Gen. Physiol., 38, 361.

Kaltenbach, J.C. (1949). Local metamorphosis of Rana Pipiens larvae by thyroxin-cholesterol implants. Anat. Rec., 103, 128.

Karlson, P. (1963). New concepts on the mode of action of hormones. Perspectives in Biol. and Med., 6, 203.

Karlson, P. and Schulz-Enders, A. (1963). Uber die Wirkung des Thyroxine auf die oxydative Phosphorylierung in Insektenmitochondrien. Gen. Comp. Endocrin., 3, 111.

Kendall, E.C. (1915). The isolation in crystalline form of the compound containing iodine which occurs in the thyroid; its' chemical nature and physiological activity. Trans. A. Am. Physicians, 30, 420.

Kernan, R.P. (1961). Insulin and the membrane potential of frog sartosius muscle. Biochem. J., 80, 23P.

Keynes, R.D. (1954). The ionic fluxes in frog muscle. Proc. Roy. Soc. B., 142, 359.

Kirschner, L.B., Maxwell, R. and Fleming, D. (1960). Non-osmotic water movement across the isolated frog skin. J. Cell. Comp. Physiol., 55, 267.

Kobayashi, H. and Gorbman, A. (1962). Thyroid function in Amphiuma. Gen. Comp. Endocrin., 2, 279.

Koch, H.J. and Heuts, M.J. (1942). Influence de l'hormone thyroidienne sur la regulation osmotique chez Gasterosteus aculeatus L. Ann. Soc. Roy. Zool. de Belgique, 73, 165.

Koefoed-Johnsen, V. (1955). quoted by Ussing, H.H. Ion transport across biological membranes, in 'Ion Transport across Membranes', H.T. Clark and D. Nachmansohn, eds. p. 3. Academic Press, N.Y.

Koefoed-Johnsen, V., Levi, H. and Ussing, H.H. (1952). The mode of passage of chloride ions through the isolated frog skin. Acta Physiol. Scand., 25, 150.

Koefoed-Johnsen, V. and Ussing, H.H. (1949). The influence of the corticotrophic hormone from ox on the active salt uptake in the axolotl. Acta Physiol. Scand., 17, 38.

Koefoed-Johnsen, V. and Ussing, H.H. (1953). The contributions of diffusion and flow to the passage of D2O through living membranes. Effect of neurohypophyseal hormone on isolated Anuran skin. Acta Physiol. Scand., 28, 60.

Koefoed-Johnsen, V. and Ussing, H.H. (1956). The nature of the frog skin potential. Abstr. Comm. 10th Intern. Physiol Congr., Brussels, p. 511.

Koefoed-Johnsen, V. and Ussing, H.H. (1958). The nature of the frog skin potential. Acta Physiol. Scand., 42, 298.

Koefoed-Johnsen, V. and Ussing, H.H. (1960). Ion transport. In 'Mineral Metabolism', C.L. Comar and F. Bronner, eds. p. 169. Academic Press, N.Y.

Koefoed-Johnsen, V., Ussing, H.H. and Zerahn, K. (1952). The origin of the short-circuit current in the adrenalin stimulated frog skin. Acta Physiol. Scand., 27, 38.

Kostyo, J.L. and Engel, F.L. (1960). In vitro effects of growth hormone and corticotrophin preparations on amino acid transport by isolated rat diaphragms. Endocrinology, 67, 708.

Krahl, M.E. (1961). The Action of Insulin on Cells. Academic Press, N.Y.

Krebs, H.A. (1954). In 'Chemical Pathways of Metabolism' volume 1. D.N. Greenberg, ed. Academic Press, N.Y.

Krogh, A. (1937). Osmotic regulation in the frog (R. esculenta) by active absorption of chloride ions. Scand. Arch. Physiol., 76, 60.

Krogh, A. (1938). The active absorption of ions in some fresh-water animals. Z. vergl. Physiol., 25, 335.

Krogh, A. (1939). Osmotic regulation in aquatic animals. Cambridge University Press.

Kruhøffer, P.W., Thaysen, J.H. and Thorn, N.A. (1960). The alkali metals in the organism. Handb. exp. Pharmak., 30, 196. Springer-Verlag, Berlin.

Lassen, U.V. and Thaysen, T. (1961). Correlation between sodium transport and oxygen consumption in isolated renal tissue. Biochem. biophys. Acta. 47, 616.

Leaf, A. (1955). Ion transport by the isolated bladder of the toad. Resumes des communications. Third Intern. Congr. Biochem. Brussesl.

Leaf, A. (1960a). Some actions of neurohypophyseal hormones on a living membrane. J. Gen. Physiol., 43, 175.

Leaf, A. (1960b). Studies of renal tubular processes in vitro in a living membrane. Univ. Mich. med. Bull., 26, 239.

Leaf, A. (1961). Some observation on transport across the toad bladder in vitro. In 'Membrane Transport and Metabolism', A. Kleinzeller and A. Kotyk, eds. p. 247. Academic Press, N.Y.

Leaf, A. (1963). Quoted by Goodfriend T. and Kirkpatrick, J. (1963). Effects of neurohypophyseal hormones on oxidative metabolism of the toad bladder in vitro. Endocrinology, 72, 742.

Leaf, A., Anderson, J. and Page, L.B. (1958). Active sodium transport by the isolated toad bladder. J. Gen. Physiol 41, 657.

Leaf, A. and Dempsey, E. (1960). Some effects of mammalian neurohypophyseal hormones on metabolism and active transport of sodium by the isolated toad bladder. J. biol. Chem., 235, 2160.

Leaf, A. and Hays, R.M. (1961). The effect of neurohypophyseal hormones on permeability and transport in a living membrane. Rec. Prog. Hormone Res., 17, 467.

Leaf, A. and Hays, R.M. (1962). Permeability of the isolated toad bladder to solutes and its modification by vasopressin. J. Gen. Physiol., 45, 921.

Leaf, A., Page, L.B. and Anderson, J. (1959). Respiration and active sodium transport of isolated toad bladder. J. biol. Chem., 234, 1625.

Leaf, A. and Renshaw, A. (1957). Ion transport and respiration of isolated frog skin. Biochem. J., 65, 82.

Lehninger, A.L. (1956). Physiology of mitochondria; relationships between mitochondrial structure, oxidative phosphorylation, and the action of thyroxine. In 'Enzymes; Units of Biological Structure and Function', O.H. Gaebler, ed. p. 217. Academic Press, N.Y.

Lehninger, A.L. (1960). Thyroxine and the swelling and contraction cycle in mitochondria. Ann. N.Y. Acad. Sci., 86, 484.

Lehninger, A.L. (1962). Water uptake and extrusion by mitochondria in relation to oxidative phosphorylation. Physiol. Revs., 42, 467.

Lehninger, A.L. and Ray, B.L. (1957). Action of thyroxine on mitochondria. Science, 125, 748.

Lehinger, A.L., Wadkins, C.L. and Remmert, L.F. (1958). Control points in phosphorylating respiration and the action of a mitochondrial "respiration-releasing factor". Proc. Ciba. Symp. on Regulation of Cell Metabolism. G.E.W. Wolstenholme and C.M. O'Conner, eds. p. 130. J. & a. Churchill Ltd., London.

Leloup, J. and Fontaine, M. (1960). Iodine metabolism in vertibrates. Ann. N.Y. Acad. Sci., 86, 316.

Levine, R. (1957). On the mechanisms of action of hormones on cells. Survey of Biol. Progr., 3, 185.

Levine, R. and Goldstein, M.S. (1955). On the mechanism of action of insulin. Rec. Progr. Hormone Res., 11, 343.

Levine, R., Goldstein, M.S., Huddlestun, B. and Klein, S.P. (1950). Action of insulin on the permeability of cells to free hexones, as studied by its effect on the distribution of galactose. Am. J. Physiol., 163, 70.

Levine, R., Goldstein, M.S., Klein, S.P. and Huddlestun, B. (1949). The action of insulin on the distribution of galactose in eviscerated nephrectomized dogs. J. Biol. Chem., 179, 985.

Levinsky, N.G. quoted by Sawyer, W.H. (1956). The hormonal control of water and salt-electrolyte metabolism with special reference to the Amphibia. Mem. Soc. Endocrin., 5, 44.

Levinsky, N.G. and Davidson, D.G. (1957). Renal action of parathyroid extract in the chicken. Am. J. Physiol., 191, 530.

Levinsky, N.G. and Sawyer, W.H. (1952). Influence of the adenohypophysis on the frog water-balance response. Endocrinology, 51, 110.

Levinsky, N.G. and Sawyer, W.H. (1953). Significance of the neurohypophysis in regulation of fluid balance in the frog. Proc. Soc. exp. Biol. Med. N.Y. 82, 272.

de Lima, T. and Lockett, M. (1963). An investigation of the actions of aldosterone on perfused cat kidneys, by ureteric stop-flow analysis. Arch. Int. Physiol. Biochem., 71, 83.

Lindberg, O. and Ernster, L. (1954). Chemistry and physiology of mitochondria and microsomes. Protoplasmologia Monographs, Vol. III/A/4. Springer, Vienna.

Linderholm, H. (1952). Active transport of ions through frog skin with special reference to the action of certain diuretics. Acta Physiol., Scand., 27, suppl. 97.

Lindsey, A.J. (1952). Vibrating micro-electrodes in polarography. The relationship between diffusion current and frequency and amplitude of vibration. J. Phys. Chem., 56, 439.

Ling, R.W. and Gerard, R.W. (1949). The normal membrane potential of frog sartorius fibre. J. Cell. Comp. Physiol., 34, 383.

Lipsett, M.B., Schwartz, I.L. and Thorn, N.A. (1961). Hormonal control of sodium, potassium, chloride and water metabolism. In 'Mineral Metabolism', C.L. Comar and F. Bronner, eds. p. 473. Academic Press, N.Y.

Longmuir, I.S. (1954). Respiration rate of bacteria as a functio of oxygen consumption. Biochem. J., 57, 81.

Lynn, W.G. and Wachowski, H.E. (1951). The thyroid gland and its function in cold-blooded vertebrates. Quart. Rev. Biol 26, 123.

McAfee, R.D. and Locke, W. (1961). Effects of certain steroids on bioelectric current of isolated frog skin. Am. J. Physiol., 200, 797.

McDougall, E.J. and Verzar, F. (1935). Die Resorption von Wasser Aus Kochsalz und Zuckerlosungen. Pflug. Arch. ges. Physiol., 236, 321.

Maetz, J., Jard, S. and Morel, F. (1958). Action de l'aldosterone sur le transport actif de sodium de la peau de genouille. C. R. Acad. Sci., 247, 516.

Maetz, J. and Julien, M. (1961). Action of neurohypophyseal hormones on the sodium fluxes of a fresh water teleost. Nature, 189, 152.

Maffly, R.H. and Edelman, I.S. (1963). The coupling of short-circuit current to metabolism in the urinary bladder of the toad. J. Gen. Physiol., 46, 733.

Maffly, R.H., Hays, R.M., Lamdin, E. and Leaf, A. (1960). The effect of neurohypophyseal hormones on the permeability of the toad bladder to urea. J. Clin. Invest., 39, 630

Magnue-Levy, A. (1995). Ueber den respiratorischen Gaswechsel unter dem Einfluss der Thyreoidea sowie unter verschiedenen pathologischen Zustanden. Berl. Klin. Wchnschr., 32, 650.

Majno, G. and Palade, G.E. (1961). Studies on inflammation. I. The effect of histamine and serotonin on vascular permeability: an electron microscopic study. J. biophys. Biochem. Cytol., 11, 571.

Majno, G., Palade, G.E. and Schoefl, G.I. (1961). Studies on inflammation. II. The site of action of histamine and serotonin along the vascular tree: a topographical study. J. Biophys. Biochem. Cytol., 11, 607.

Mansfeld, G. (1935). Art und ort der Thyroxinwirkung. Klin. Wschr., 25, 884.

Martin, K. and Burgen, A.S.V. (1962). Changes in the permeability of the salivary gland caused by sympathetic stimulation and by catecholamines. J. Gen. Physiol., 46,225

Martius, C. and Hess, B. (1951). The mode of action of thyroxine Arch. Biochem. Biophys., 33, 486.

Matty, A.J. (1954). Thyroidectomy of the dogfish, Scyllium canicula (L), and the effect of dogfish thyroid upon the oxygen consumption of rats. J. mar. biol. Ass. U.K., 33, 689.

Matty, A.J. (1957). Thyroidectomy and its effect upon oxygen consumption of a teleost fish, Pseudoscarus guacamaia. J. Endocrin., 15, 1.

Matty, A.J. (1960). Thyroid cycles in fish. Symp. Zool. Soc. Lond., 2, 1.

Matty, A.J. and Green, K. (1962a). Active sodium transport in response to thyroxine. Life Sciences, 1, 487.

Matty, A.J. and Green, K. (1962b). Thyroid hormones and membrane permeability. Abstr. Comm. 1º Comp. Endocrin. Conf., London. Gen. Comp. Endocrin. 2, suppl. p. 616.

Matty, A.J. and Green, K. (1963a). Permeability and respiration effects of thyroidal hormones on the isolated bladder of the toad, Bufo bufo. J. Endocrin., 25, 411.

Matty, A.J. and Green, K. (1963b). The effect of thyroidal hormones on water permeability of the isolated bladder of the toad, Bufo bufo. J. Endocrin., 26, Proc. Endocrin., Soc., 91, xxii.

Matty, A.J. and Green, K. (1963c). Thyroid hormones and membrane permeability. Abstr. Comm. 2º Comp. Endocrin., Conf. Brussels., Abstr. 53.

Meier, A.H. and Fleming, W.R. (1962). The effects of Pitocin and Pitressin on water and sodium movements in the euryhaline killifish, Fundulus kansae. Comp. Biochem. Physiol 6, 215.

Menozzi, P.G. and Gatto, E. (1961). Effecto della tiroxine sul volume di distribuzions endocellulare del D (+) xiloso e del sodio nel tessuto adiposo epidimario di ratto. Arch. Maragliano, 17, 1101.

Mitchell, P. (1961). Biological transport phenomena and the spacially anisotropic characteristics of enzyme systems causing a vector component of metabolism. In 'Membrane Transport and Metabolism' A. Kleinzeller and A. Kotyk, eds. p. 100. Academic Press, N.Y.

Mitchell, P. (1962). Molecule, group and electron translocation through natural membranes. Biochem. Soc. Symp., 22,142

Morel, F. (1962). Physiologie des hormones neurohypophysaires. Ext. de la Biol. Med., 51, 152.

Muller, J. (1953). Uber die Wirkung von Thyroxin und Thyriotropem Hormon auf den Stoffwechel und die Farbung der Goldfisches. Z. vergl. Physiol., 35, 1.

Myant, N.B. (1963). The thyroid gland and the metabolism of lipid Postepy Biochemii, 9, 3.

Nasset, E.S. and Goldsmith, D.P.J. (1961). Effect of thyroid on gastric secretion and metabolism. Am. J. Physiol., 201, 567.

Orloff, J. and Handler, J.S. (1962). The similarity of effects of vasopressin, adenosine-3'-5'-phosphate (cyclic AMP) and theophylline on the toad bladder. J. Clin. Invest., 41, 702.

Paget, G.E. and Thorp, J.M. (1963). An effect of thyroxine on the fine structure of the rat liver cell. Nature, 199, 1307.

Park, C.R., Johnson, L.H., Wright, J.H. and Batsel, H. (1957). Effect of insulin on transport of several hexoses and pentoses into cells of muscle and brain. Am. J. Physiol., 191, 13.

Parsons, D.E. and Wingate, D.L. (1961). The effect of osmotic gradients on fluid transfer across rat intestine in vitro. Biochem. biophys. Acta, 46, 170.

Penney, D.E., McAfee, R.D. and Locke, W. (1961). The effect of aldosterone and aldosterone inhibitor SC 8109 on sodium transport in the isolated frog skin. Fed. Proc., 21,413

Pickford, G.E. and Atz, J.W. (1957). The Physiology of the Pituitary Gland of Fishes. New York Zoological Society

Pitt-Rivers, R. (1953). Physiological activity of the acetic acid analogues of some iodinated thyronines. Lancet, 11, 234

Pitt-Rivers, R. and Tata, J.R. (1959). The Thyroid Hormones. Pergamon Press, London.

Post, R.L. (1961). Sodium and potassium transport across the human erythrocyte membrane. In 'Biophysics of Physiological and Pharmacological Actions' p. 19 Am. Assoc. for Adv. of Sci., Washington.

Pritchard, A.W. and Gorbman, A. (1960). Thyroid hormone treatment and oxygen consumption in embryos of the spiny dogfish. Biol. Bull., 119, 109.

Radsma, W., Golterman, H.L. and Birkenhager, J.C. (1954). The influence of thyroxine on the oxygen consumption of liver homogenates. Biochem. biophys. Acta, 14, 150.

Randle, P.J. and Morgan, H. (1962). Regulation of glucose uptake by muscle. Vitamins and Hormones, 20, 199.

Rasmussen, H. (1959). The influence of parathyroid function upon the transport of calcium in isolated sacs of rat small intestine. Endocrinology, 65, 517.

Rasmussen, H., Schwartz, I.L., Schoessler, M.A. and Hochster, G. (1960). Studies on the mechanism of action of vasopressin. Proc. nat. Acad. Sci., Wash., 46, 1278.

Rasmussen, H., Schwartz, I.L., Young, R. and Marc-Aurele, J. (1963) Structural requirements for the action of neurohypophyseal hormones upon the isolated amphibian urinary bladder. J. Gen. Physiol., 46, 1171.

Rawson, R.W., Rall, J.E., Pearson, O.H., Robbins, J., Poppell, H.F. and West, R.D. (1953). L-Triiodothyronine versus L-thyroxine, a comparison of their metabolic effects in human myxedema. Am. J. med. Sci., 226, 405.

Rehm, W., Schuman, H. and Heinz, E. (1961). Insulin on frog gastric mucosa. Fed. Proc., 21, 193.

Reinwein, H. and Singer, W. (1928). Studien der Gewebratmung. IV. Der Einfluss von Thyroxin, Adrenalin und Insulin auf den Fauerstoffverbrauch uberlebender Leberzellen. Biochem. Ztsch., 197, 152.

Robinson, J.R. (1954). Secretion and transport of water. In 'Active Transport and Secretion'. S.E.B. Symposium No. 8, p. 42. Cambridge University Press.

Roche, J. and Michel, R. (1960-. The peripheral metabolism of thyroid hormones. Ann. N.Y. Acad. Sci., 86, 454.

Roche, J., Michel, R., Truchot, R. and Wolf, W. (1955). Sur les activites biologiques des iodothyronines et de leurs derives. C.R. Soc. Biol., 149, 1219.

Root, R.W. and Etkin, W. (1937). Effect of thyroxine on the oxygen consumption of the toadfish. Proc. Soc. exp. Biol. Med. N.Y., 37, 174.

Rosenberg, T. (1954). The concept and definition of active transport. In 'Active Transport and Secretion'. S.E.B. Symposium No. 8, p. 27. Cambridge University Press.

Rossi, G. and Capraro, V. (1961). Net transport of water and solutes by an in vitro rat intestinal preparation. Experienta, 17, 543.

Saito, T. (1930). Fol. endocrin. Jap., vi, 19. quoted by Gellhorn, E. and Northrup, D. (1933). Quantitative investigations on the influence of hormones on absorption Am. J. Physiol., 103, 382.

Satoyoshi, E., Murakami, K., Dowa, H., Kinoshita, M., Noguchi, K., Noshina, S., Nishiyama, Y. and Ito, K. (1963). Myopathy in thyrotoxicosis. Neurology, 13, 645.

Sawyer, W.H. (1956). The hormonal control of water and salt-electrolyte metabolism with special reference to the Amphibia. Mem. Soc. Endocrin., 5, 44.

Sawyer, W.H. (1960). Increased water permeability of the bullfrog (Rana catesbiana) bladder in vitro in response to synthetic oxytocin, and arginine vasotocin and to neurohypophyseal extracts from non-mammalian vertebrates. Endocrinology, 66, 112.

Sawyer, W.H. (1961a). Comparative physiology and pharmacology of the neurohypophysis. Rec. Progr. Hormone Res., 17, 437

Sawyer, W.H. (1961b). Neurohypophyseal hormones. Pharmacol. Revs 13, 225.

Sawyer, W.H. and Schisgall, R.M. (1956). Increased permeability of the frog bladder to water in response to dehydration and neurohypophyseal extracts. Am. J. Physiol., 187, 312.

Sawyer, W.H., Travis, D.F. and Levinsky, N.G. (1950). Identity and specificity of frog water balance principle of posterior pituitary extracts. Am. J. Physiol., 163, 364.

Scheer, B.T. (1958). Active transport; definition and criteria. Bull. Math. Biophys., 20, 231.

Schoffeniels, E. (1962). Isolation of a sodium-dependent ATPase from the gills of Octopus vulgaris L. Life Sciences, 1, 437.

Schoffeniels, E. and Baillien, M. (1960). Action des hormones sexuelles sur les characteres de permeabilits de la peau de grenouille. Arch. Int. Phys. Biochem., 68, 376.

Schoffeniels, E. and Tercafs, R.R. (1962). Potential difference and net flux of water in the isolated Amphibian skin. Biochem. Pharmacol., 11, 769.

Schultz, H., Low, H., Ernster, L. and Sjostrand, F.S. (1956). Elektronenmikroskopische Studien an Leberschnitten von Thyroxin-benhandelten Ratten. Proc. Stockholm Conf. on Electron Microscopy, p. 134.

Scott, A.H. (1935). Thyroxine and tissue metabolism. Am. J. Physiol., 111, 107.

Sexton, A.W. (1955). quoted by Chester Jones, I., Phillips, J.G. and Holmes, W.N. (1959). Comparative physiology of the adrenal cortex. In 'Comparative Endocrinology'. A. Gorbman, ed. p. 582. Wiley.

Sharp, G.W.G. (1963). The effect of cortisol and certain synthetic steroids on the permeability of synovial membrane in the rabbit. J. Endocrin., 25, 443.

Shellabarger, C.J. and Brown, J.R. (1959). The biosynthesis of thyroxine and 3:5:3'-triiodothyronine in larval and adult toads. J. Endocrin., 18, 98.

Siekevitz, P. (1959). In Ciba Foundation Symposium on the Regulation of Cell Metabolism. G.E.W. Wolstenholme and C.M. O'Conner, eds. p. 17. J. & A. Churchill Ltd.London

Siekevitz, P. (1961). Some problems bearing on the relationship between intracellular organelles. Symposium on Functional Biochem. of Cell Structures, Vth, Intern. Congr. of Biochem., Moscow.

Skou, J.C. (1957). The influence of some cations on an adenosinetri-phosphatase from peripheral nerves. Biochem. biophys. Acta, 23, 394.

Skou, J.C. (1960). Further investigations on a $Mg^{2+} + Na^{+}$-activated adenosinetriphosphatase, possibly related to the active linked transport of Na^{+} and K^{+} across the nerve membrane. Biochem. biophys. Acta, 42, 6.

Skou, J.C. (1961). The relationship of a $(Mg^{++} + Na^{+})$-activated, K^{+}-stimulated enzyme or enzyme system to the active, linked transport of Na^{+} and K^{+} across the cell membrane. In 'Membrane Transport and Metabolism', A. Kleinzeller and A. Kotyk, eds. p. 228. Academic Press, N.Y.

Skou, J.C. (1962). Preparation from mammalian brain and kidney of the enzyme system involved in active transport of Na^{+} and K^{+}. Biochem. biophys. Acta, 58, 314.

Sokoloff, L., Campbell, P.L., Francis, C.M. and Gelboin, H.N. (1962) Thyroxine stimulation of amino acid incorporation into protein: localisation of stimulated step. Fed. proc., 21, 217.

Sokoloff, L. and Kaufman, S. (1959). Effect of thyroxine on amino acid incorporation into protein. Science, 129, 569.

Solomon, D.H. (1961). Effects of thyrotropin on thyroidal water and electrolytes in the chick. Endocrinology, 69, 939

Smith, D.C. and Everett, G.M. (1943). The effect of thyroid hormone on growth rate, time of sexual differentiation and oxygen consumption in the fish, Lebistes reticulatus. J. exp. Zool., 94, 229.

Smith, D.C. and Matthews, S.A. (1948). Parrot fish thyroid extract and its effect upon oxygen consumption in the fish, Bathystoma. Am. J. Physiol., 153, 215.

Smith, D.C.W. (1956). The role of the endocrine organs in the salinity tolerance of trout. Mem. Soc. Endocrin., 5, 83.

Smith, H.W. (1930). Metabolism of the lung-fish, Protopterus aethiopicus. J. biol. Chem., 88, 97.

Smyth, D.H. and Taylor, C.D. (1954). Transport of water and other substances through the intestinal wall. J. Physiol., 126, 42P.

Stassilli, N.R., Kroc, R.L. and Meltzer, R.I. (1959). Antigoitrogenic and calorigenic activities of thyroxine analogues in rats. Endocrinology, 64, 62.

Stephan, F., Jahn, H. and Maetz, B. (1959a). Action de l'insuffisance thyroidienne sur l'elimination urinaire de l'eau, du sodium et du potassium chez le rat. C.R. Soc. Biol., 153, 332.

Stephan, F., Jahn, H. and Maetz, B. (1959b). Action de l'insuffisance thyroidienne sur l'excretion renale de l'eau, du sodium et du potassium chez le Rat. C.R. Acad. Sci., 248, 1227.

Stephan, F., Jahn, H. and Reville, P. (1960). Action de l'insuffisance thyroidienne sur les echanges hydrique du Rat. C.R. Soc. Biol., 154, 1082.

Stephan, F., Jahn, H., Reville, P. and Urban, M. (1961). Effet de l'insuffisance thyroidienne sur la concentration de l'uree, due sodium et du potassium dans les urines du Rat. C.R. Soc. Biol., 155, 1555.

Szego, C.M. and Roberts, S. (1953). Steroid action and interaction in uterine metabolism. Rec. Progr. Hormone Res., 8,419.

Tapley, D.F. (1955). Magnesium balance in myxedematous patients treated with triiodothyronine. Johns Hopk. Hosp. Bull., 96, 274.

Tapley, D.F. (1956). The effect of thyroxine and other substances on the swelling of isolated rat liver mitochondria. J. Biol. Chem., 222, 325.

Tapley, D.F. (1962). The mechanism of action of thyroid hormones. Am. Zool., 2, 373.

Tapley, D.F. and Cooper, C. (1956). The effect of thyroxine and related compounds on oxidative phosphorylation. J. hiol. Chem., 222, 341.

Tapley, D.F., Cooper, C. and Lehninger, A.L. (1955). The action of thyroxine on mitochondria and oxidative phosphorylation. Biochem. biophys. Acta, 18, 597.

Tapley, D.F. and Hatfield, W.B. (1962). The peripheral action of thyroxine. Vitamins and Hormones, 20, 251.

Tata, J.R. (1961). Is thyroid hormone metabolism related to its action? Mem. Soc. Endocrin., 11, 90.

Tata, J.R. (1962). Intracellular and extracellular mechanism for the utilisation and action of thyroid hormones. Rec. Progr. Hormone Res., 18, 221.

Tata, J.R., Ernster, L. and Lindberg, O. (1962). Control of basal metabolic rate by thyroid hormone and cellular function Nature, 193, 1058.

Tata, J.R., Ernster, L., Lindberg, O., Arrhenius, E., Pederson, S. and Hedman, R. (1963). The action thyroid hormones at the cell level. Biochim. J., 86, 408.

Taubenhaus, M., Fritz, I.B. and Morton, J.V. (1956). *In vitro* effects of steroids upon electrolyte transfer through frog skin. Endocrinology, 59, 458.

Taurog, A. and Chaikoff, I.L. (1948). On the nature of plasma iodine. J. biol. Chem., 171, 439.

Taurog, A., Wheat, J.D. and Chaikoff, I.L. (1956). Nature of the I^{131} compounds appearing in the thyroid vein after injection of iodine -I^{131} Endocrinology, 58, 121.

Taylor, A. (1936). Athyroidism in the salamander, *Triturus torosus* Rathke. J. exp. Zool., 73, 153.

Taylor, A. (1939). The effect of athyroidism and hyperthyroidism on the oxygen consumption of the adult salamander. J. exp. Zool., 81, 135.

Tepperman, J. and Tepperman, H.M. (1960). Some effects of hormone on cells and cell constituents. Pharmacol. Revs., 12, 301.

Tercafs, R.R. (1963). Comparative study of the action of some pharmacological substances on chromatophores and on permeability characteristics of amphibian skin. Abstr. Comm. 20. Europ. Conf. Comp. End., Brussels, p. 55.

Thibault, O. (1956). Action de l'acide triiodothyroacetique sur le metabolisme de base du rat ethyroide. Ann. Endocrinl, Paris, 17, 35.

Thibault, O. and Pitt-Rivers, R. (1955). Immediate effects of thyroxine analogues on biological oxidations *in vitro*. Lancet, i, 285.

Thompson, W. (1925-26). Studies in blood volume. J. Clin. Invest. 2, 477.

Thornburn, C.C. and Matty, A.J. (1963). The effect of thyroid hormones *in vitro* on the oxygen consumption of toad tissues. J. Endocrin., in press.

Tomita, K., Lardy, H.A., Larson, F.C. and Albright, E.C. (1957). Enzymatic conversion of thyroxine to tetraiodothyroacetic acid and of triiodothyronine to triiodothyroacetic acid. J. biol. Chem., 224, 387.

Tonoue, T. (1962). Relation between ATP concentration and effect of thyroxine on oxygen consumption rate of liver homogenate. Endocrinology, 70, 68.

Ullrich, K.J. and March, D.J. (1963). Kidney, water and electrolyte metabolism. Ann. Rev. Physiol., 25, 91.

Ungar, I. (1933). La cause de la production d'une pellicule cutanee chez le crapaud hypophysoprive ou a tuber lese. C.R. Soc. Biol., 112, 504.

Ussing, H.H. (1948). The use of tracers in the study of active ion transport across animal membranes. Cold Spring Harbor Symp. Quant. Biol., 8, 193.

Ussing, H.H. (1949a). The active ion transport through frog skin in the light of tracer studies. Acta Physiol. Scand., 17, 1.

Ussing, H.H. (1949b). The distinction by means of tracers between active transport and diffusion. Acta Physiol. Scand., 19, 43.

Ussing, H.H. (1949c). Transport of ions across cellular membranes Physiol. Revs., 29, 27.

Ussing, H.H. (1955). General principles and theories of membrane transport. In 'Metabolic Aspects of Transport across Cell Membranes', H.T. Clark, ed. p. 39. Univ. Wisconsin Press, Madison.

Ussing, H.H. (1959). Active and passive transport actoss epithelial membranes. In 'The Method of Isotopic Tracers Applied to the Study of Active Ion Transport', J. Coursaget, ed. p. 139. Pergamon Press, London.

Ussing, H.H. (1960a). Ion transport mechanisms. In 'Biological Approaches to Cancer Chemotherapy', p. 89. Academic Press, N.Y.

Ussing, H.H. (1960b). The frog skin potential. J. Gen. Physiol., 43, 135.

Ussing, H.H. (1960c). The alkali metal ions in isolated systems and tissues. Handb. exp. Pharmak., 30, 1. Springer-Verlag, Berlin.

Ussing, H.H. and Anderson, B. (1955). The relation between solven drag and active transport of ions. Proc. Intern. Congr. Biochem., 3 Congr., Brussels, p. 434.

Ussing, H.H. and Zerahn, K. (1951). Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. Acta Physiol. Scand., 23, 110.

Verebely, T.v. (1932). Der mechanismus der Thyroxinwirkung. Klin. Wschr., 11, 1705.

Warren, M.R. (1940). Studies on the effect of experimental hyperthyroidism on the adult frog, Rana pipiens Schreber. J. exp. Zool., 83, 127.

Weinstein, E.J. and Lein, A. (1957). Response of isolated rat diaphragm to thyroxine in vitro. Endocrinology, 61, 79

Weiss, A.K. (1956). Effects of in vitro administration of thyroxine and triiodothyronine on surviving liver slices. Am. J. Physiol., 185, 243.

Wheeler, K.P. and Whittam, R. (1962). Some properties of a kidney adenosine triphosphatase relevant to active cation transport. Biochem. J., 85, 495.

Whittembury, G., Oken, D.E., Windhager, E.E. and Solomon, A.K. (1959). Single proximal tubules of the Necturus kidney IV. Dependence of H_2O movement on osmotic gradients. Am. J. Physiol., 197, 1121.

Wijdeveld, P.G.A.B. and Jansen, A.P. (1960). Renal concentrating and water excreting capacity in hyperthyroidism. Clin. Chem. Acta, 5, 618.

Wilbrandt, W. (1963). Aktiver Transport durch Grenzflaschen. Klin. Wschr., 41, 138.

Williams, M.W. and Angerer, C.A. (1959). Adrenal cortex and frog skin potentials. Proc. Soc. exp. Biol. Med. N.Y. 102, 112.

Windhager, E.E. and Giebisch, G. (1961). Comparison of short-circuit current and net water movement in single perfused proximal tubules of rat kidneys. Nature, 191, 120

Windhager, E.E., Whittembury, G., Oken, D.E., Schatzmann, H.J. and Solomon, A.K. (1959). Single proximal tubules of the Necturus kidney. III. Dependence of H_2O movement on NaCl concentration. Am.J. Physiol., 197, 313.

Wiswell, J.G. and Asper, S.P. (1958). Studies of thyroxine and some of its analogues. V. Metabolic activity in vitro and in vivo of the acetic acid analogues of triiodothyronine and thyroxine. Johns Hopk. Hosp. Bull., 102, 115.

Wiswell, J.G., Zierler, K.L., Fasano, M.B. and Asper, S.P. (1954). The effects of L-triiodothyronine and L-thyroxine on the metabolism of tissues in vitro. Johns Hopk. Hosp. Bull., 94, 94.

Wool, I.G. and Manchester, K.L. (1962). Insulin and incorporation of amino-acids into protein of rat tissues. Nature, 193, 345.

Yoshihiro, M. (1956). Effect of thyroxine upon the oxygen consumption of dog adrenal cortex in vitro. Gunma J. Med. Sci., 5, 37.

Zerahn, K. (1956). Oxygen consumption and active sodium transport in the isolated and short-circuited frog skin. Acta. Physiol., Scand., 36, 300.

Zerahn, K. (1958). Oxygen consumption and active sodium transport in isolated amphibian skin under varying experimental conditions. Universitetsforlaget i Aarhus.

Zerahn, K. (1961). Active sodium transport across isolated frog skin in relation to metabolism. In 'Membrane Transport and Metabolism', A. Kleinzeller and A. Kotyk, eds, p. 237. Academic Press, N.Y.

Zierler, K.L. (1956). Effect of insulin on membrane potential and potassium content of rat muscle. Am. J. Physiol., 197, 515.

Zyle, A.v. and Engelbrecht, F.M. (1959). The oxygen uptake of kidney cortex slices of thyroidectomised rats as influenced by triiodothyroacetic acid. J. Endocrin., 19, 108.